ORIGIN OF THE MESODERM AND BEHAVIOR OF THE NUCLEOLUS IN REGENERATION IN *LUMBRICULUS*.[1]

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Introduction.

The microdrilous annelids have been the center of considerable interest on the part of students of regeneration particularly from the point of view of the origin of the cells which form the new tissue. While the production of new endodermal and ectodermal structures from cells of the corresponding old parts is generally accepted, the origin of the mesoderm in the bud has been a point of contention. Hepke ('97) in Naids, von Wagner ('00 and '06) in *Lumbriculus* and Abel ('02) in *Tubifex* and *Nais*, among others, are of the opinion that the new mesodermal structures both in anterior and in posterior regeneration are of ectodermal origin. Randolph ('92) in posterior regeneration in *Lumbriculus* reports their formation from comparatively unmodified cells of the old mesoderm to which she gives the name "neoblasts." Iwanow ('03) in *Lumbriculus* and Krecker ('10) in *Tubifex* and *Limnodrilus* verify her conclusions. They find that in anterior regeneration, however, cells from old specialized mesodermal structures form the new portion. One of the features in the descriptions of the cells of the bud region by these workers is the prevalence of large nucleoli in the cells involved in the regenerative activity. Krecker ('23) gives a rather complete description of the origin and migration of the neoblasts in posterior regeneration. Studies on the origin of the new mesoderm in regeneration together with observations on nucleolar changes in these and other tissues are reported in this paper. For invaluable advice on this work I am indebted to Dr. J. W. Wilson of Brown University at whose suggestion the problem was first undertaken.

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Materials and Methods.

The species used in this work was identified for me by Professor Frank Smith, of the University of Illinois, as of the genus *Lumbriculus*, and probably the species *inconstans* Smith. Anæsthetization was avoided as far as possible. When it was necessary, a one eighth of one per cent. solution of chloretone or a four per cent. solution of alcohol, redistilled in glass, gave equally satisfactory results. For histological fixation Zenker's fluid or Bouin's Picroformol were used; best results were obtained with the latter. It was not necessary to anæsthetize the worms first. An individual to be fixed was placed on a glass plate and straightened against a glass slide. Another slide was then pushed up until it came in contact with the worm, care being taken not to crush it between the slides. In this process only a very small amount of water was used. The fixing fluid was then slowly added and its surface tension, as it was drawn underneath the two slides, held the worm firmly in place during the preliminary fixation. This method is a modification of one described by Welch ('13). As a stain Heidenhain's iron alum-hematoxylin method was used for the most part, though preparations were also made with Delafield's hematoxylin and eosin, used both as a direct and as a regressive stain. On carefully controlled regressive staining the chromatin granules were stained with the hematoxylin while the nucleoli took the eosin, indicating that they were true nucleoli or plasmosomes.

Nuclei and Nucleoli in the Cells of Uninjured Individuals.

As this paper is to deal at some length with the cytological changes, particularly nuclear and nucleolar, which occur during regeneration, it is perhaps advisable first of all to discuss more or less completely the conditions which exist in uninjured individuals. Under this heading the usual appearance of cells in the hypodermis, setigerous glands and intestine, including those differences which may exist at the growing region of the tail, will be described.

Hypodermis.

In all the epithelial cells of the hypodermis the nuclei are quite large, all gradations from an oval type (the average measurement of twenty-five of which is 3.3 x 5.8 μ), to the practically spherical one (averaging about 4.2 μ) are to be found. In each a very minute nucleolus, appearing as a mere dot under a magnification of 1,000 $\times$, is visible (Fig. 1). Cells of this type form all of the hypodermis from the second segment to the growing region of the tail. At this end, just anterior to the place where the basement membrane is discontinuous due to the formation of the ventral nerve cord, the hypodermal cells are slightly elongated. The nucleoli are slightly larger than in the cells located more anteriorly (Fig. 2). Cells of this type are probably intermediate stages between the active ones of the growing region and the typical hypodermal cells found in the older part of the body. Such an elongation of the cells becomes even more marked where the nerve cord is forming with the result that the hypodermis in the growing tip is two or three times as thick as in the rest of the body wall. This elongation of the cells is most marked ventrally just posterior to the end of the nerve cord. The thickening of the hypodermis includes the dorsal and lateral portions as well as the ventral but it does not extend as far anteriorly on the dorsal side as elsewhere.

Bülow ('83), in his discussion of the normal growing region, describes this thickening, but makes no mention of any difference in the nucleoli of this region. In posterior regeneration, however, Krecker ('10) describes a marked enlargement of the nuclei and nucleoli of the ventral ectoderm. In the normal growing worm the nuclei are larger near the growing nerve cord and a short distance posterior to it than they are elsewhere (Fig. 2); the average size of these nuclei is 3.9 x 7.4 μ. The cells in which such a difference is apparent are the ones involved in the formation of the new nerve cord. The nucleoli, on the other hand, are enlarged throughout the entire tip and to a slight degree for a short distance from it. This difference is most decided in the ventral cells, in which the nucleoli may be nearly 2 μ in diameter, but average between 1 and $1\frac{1}{2}$ μ.

Setigerous Glands.

Each pair of setæ is embedded in a mass of cells derived from the ectoderm and to them are attached secondarily mesodermal cells which form their musculature (Penners, '23). The setæ themselves are formed by the ectoderm cells, according to the description of Bergh ('90), more recently corroborated by Penners. In the earlier stages of the formation of the setigerous glands in the growing region of the tail most of the cells possess oval nuclei of about the same size as those of the neighboring ectoderm cells and nucleoli measuring between 1 and 1½ μ. As muscle fibers are added to the outside of the gland cells it becomes more difficult to study the latter but it seems certain that most of them have large nucleoli for a number of segments from the growing region. In the older part of the worm, however, the nucleoli are smaller, for the most part mere dots (Fig. 3). Occasionally a few cells are to be found possessing fairly large nucleoli (1 μ); these cells may have been active in the formation of a new seta to replace one which had been lost.

Alimentary Canal.

The cells of the alimentary canal in the first segment are typical hypodermal cells, of cuboidal shape with round nuclei 4 to 5 μ in diameter and nucleoli which appear as mere dots (Fig. 1). These cells, unlike those of the rest of the digestive tract, are without cilia but covered with a cuticle, as are those of the hypodermis (Pointer, '11). In the second somite the cells are more elongated and the nuclei are of the oval shape (3.6 x 6.9 μ) typical of most gut cells of this species. This segment is a transition zone between the first and third as far as the nucleoli are concerned. In the latter the nucleoli are fully ½ μ in diameter (Fig. 4).

These same measurements hold for both the nuclei and the nucleoli in the cells of the next few segments. At about the ninth or tenth segment, however, cells with larger nucleoli appear. These cells increase in abundance until in the vicinity of the thirteenth practically every nucleolus measures at least 1 μ (Fig. 5). There is no corresponding enlargement of nuclei. A feature which first appears in this region is the occurrence of

two nucleoli within a single nucleus. This is a phenomenon which is rather infrequent in this species except in regenerating individuals. For brevity, they will be spoken of as "double nucleoli" but this meaning of the words should not be confused with that of earlier workers, particularly Montgomery ('98), whose "double nucleoli" were individual nucleoli made up of two types of material. While no unquestionable cases of double nucleoli have been observed in the first eleven segments, posterior to this region, where the nucleoli are larger, cases of this sort are occasionally found. Counts made on several uninjured individuals indicate that the average frequency is about 3 per segment.

This larger type of nucleolus is typical of the gut cells for a considerable distance. Then there is a gradual decrease in size. In some individuals it seems to be at considerable distance from the anal segment—even 30 segments or more—while in others the larger nucleoli apparently persist at least 15 or 20 segments farther. In this posterior portion of the gut they are hardly more than mere dots in the nuclei (Fig. 2).

Mesoblasts.

In the ventral portion of the cœlom in the growing tail region, cells are present which are presumably derived from the mesoblasts of the developing individual (Wilson, '89 and '92). These cells, which produce the mesodermal structures in the newly forming segments, possess large nuclei (about 4 x 8 μ) and nucleoli (about 2 μ) (Fig. 2).

Large nucleoli are present, therefore, in the cells of the middle portion of the gut, in those of the hypodermis of the growing tail region and in the mesoblasts. The largest of these are in the cells of the ventral region of the ectoderm, which are forming the new nerve cord, and in the mesoblasts, from which the new mesodermal structures are derived. The significance of these facts will be discussed under a later heading.

Origin of New Tissue in Posterior Regeneration.

Endoderm.

In posterior regeneration in microdrilous annelids the majority of investigators, especially more recently, are agreed on the method of origin of the endodermal structures. Krecker ('10) in *Tubifex* and *Limnodrilus* removes a portion of the intestine after cutting a worm and finds that by a proliferation of cells in the old intestine growth takes place until it comes in contact with the ectoderm. He says (p. 411) that "mitosis and amitosis both occur," using as a criterion for the latter "the frequent occurrence of double and elongating nucleoli." He finds that cell division may take place one or two segments anterior to the wound, but when it "was seen at such a distance it was always found to be mitotic . . . ." His observations are verifications of statements to the same effect made by Iwanow ('03). A study of individuals which have been regenerating for various periods reveals that mitoses may and commonly do occur even eleven or twelve segments from the wound region. The distribution of mitotic figures and double nucleoli in worms regenerating for different periods is given in Tables I. to VI., inclusive.

Table I.

1 Day of Regeneration.

Segment from the wound	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Double nucleoli	8	8	7	7	10	6	4	4	5	3	2	2	2	3
Mitoses	0	0	0	0	1	0	0	0	0	0	0	0	0	0

Table II.

2 Days of Regeneration.

Segment from the wound	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Double nucleoli	25	31	20	25	21	25	23	20	22	23	19	18	6	2
Mitoses	10	8	4	3	7	4	3	3	2	5	2	2	0	0

Table III.

3 Days of Regeneration.

Segment from the wound	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Double nucleoli	26	21	34	25	27	23	25	21	23	17	9	9	7	3
Mitoses	14	2	5	2	5	3	3	0	2	5	3	1	1	0

Table IV.

4 Days of Regeneration.

Segment from the wound.....	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Double nucleoli.....	26	26	53	46	32	19	15	8	8	7	6	3	1	2
Mitoses.....	5	6	4	7	6	1	0	0	1	1	1	1	0	0

Table V.

5 Days of Regeneration.

Segment from the wound.....	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Double nucleoli.....	44	37	41	33	24	18	18	9	9	6	5	6	3	2
Mitoses.....	6	5	7	3	4	1	4	3	1	1	0	0	0	0

Table VI.

6 Days of Regeneration.

Segment from the wound.....	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Double nucleoli.....	20	23	18	17	20	15	16	7	5	3	4	2	1	3
Mitoses.....	3	2	4	1	1	0	1	0	0	0	1	0	0	0

In making these counts it is necessary to adopt some criterion for double nucleoli. These figures, therefore, are for cases where a nucleus contains two nucleoli in approximately the same focal plane of the microscope. In order that the personal equation involved in making the counts may be made as negligible as possible, instances of dumbbell-shaped or elongated nucleoli are not included. Records are not given for segments beyond the fourteenth from the wound since but 2 or 3 double nucleoli per segment, the number ordinarily found in an uninjured worm, are to be found in these. For mitotic figures all stages from the appearance of the chromosomes in the prophase to the late telophase are included.

As already mentioned, the gut cells of these worms ordinarily have nucleoli of considerable size, averaging about 1 μ in diameter, and the oval nuclei measure on the average about 4 x 7 μ. With the onset of regeneration an increase in the size of the nuclei and nucleoli begins, though it is comparatively slight in the former case.

Twelve hours after a worm is cut such a change in the nucleoli near the wound is apparent and by the second day many of them measure at least 2 μ in diameter (Fig. 6). Similarly by the second day the nuclei have enlarged until the average of a number measured is 5.1 x 8.8 μ. This increase in the amount of nuclear and nucleolar materials is not confined merely to the wound region but extends even eleven or twelve segments from it. Farther away than this, however, these structures show no change from the size ordinarily present in such a region (Fig. 5).

Double nucleoli are found in increased numbers within the same limits as are enlarged ones and beyond the twelfth or thirteenth segment from the wound they are no more abundant than in an uninjured individual (Tables I. to VI. inclusive). There is no gradual decrease in number, however, as one gets farther from the wound but a somewhat abrupt drop about the twelfth segment. Mitoses, too, are found only in these same twelve or thirteen segments and, though usually most numerous in the two or three segments nearest the bud, they too do not gradually decrease in number but stop rather abruptly.

TABLE VII.

NUMBER OF DOUBLE NUCLEOLI.

Number of Days of Regeneration.	Segments from Wound.		Total.
	1–5 (incl.).	6–12 (incl.).	
1	40	26	66
2	122	150	272
3	133	127	260
4	183	66	249
5	179	71	250
6	98	52	150

Double nucleoli and mitoses increase rapidly in numbers during the early part of the regeneration period and then gradually decrease again. Double nucleoli have begun to appear on the first day but only one instance of mitosis is found. There is a great increase in the number of each on the second day. By the fourth day, however, the number of double nucleoli has dropped decidedly in the segments more distant from the wound; this is counterbalanced by an increase in the five nearest segments

with the result that the total number is not appreciably changed. Table VII. gives the comparative abundance of double nucleoli in the first five segments and in the other seven which are apparently involved in the regenerative processes.

The number of mitoses also decreases in the more distant portion but in this case it is not compensated by an increase near the wound so that the total number is less. The major part of the activity of the gut in forming new tissue, therefore, is now confined to a more restricted area. Table VIII. gives data for mitoses corresponding to that given in Table VII. for double nucleoli.

TABLE VIII.

NUMBER OF MITOSES.

Number of Days of Regeneration.	Segments from Wound.		Total.
	1–5 (incl.).	6–12 (incl.).	
1	1	0	1
2	32	21	53
3	28	17	45
4	28	5	33
5	25	10	35
6	11	2	13

The records for the fifth day are approximately the same as those for the fourth. On the sixth the numbers both of double nucleoli and of mitoses have decreased decidedly throughout the entire region involved in regeneration. This decrease is most marked, however, in the more distant segments. By the seventh day the intestine of the old region has returned to approximately its usual appearance in an uninjured worm. Only two or three cases of mitosis are to be found and double nucleoli are little if any more abundant than in intact animals.

Beginning with the second or third day many cells in which the nucleoli are small are present in the intestine of the old part. These are probably the products of a recent mitotic division during which the nucleolus has been lost. That the nucleoli of these cells enlarge gradually is evidenced by the fact that the number of such cells does not increase appreciably during further regeneration. Similarly most of the cells in the newly formed gut tissue of the regenerating bud have small nucleoli at first.

By the third day these, too, are enlarging so that it is not possible to discover a line of demarcation between the gut of the bud and that of the old part. At about this time double nucleoli and mitotic figures begin to appear in the new gut. Cell proliferation in the new tissue thus increases as that in the old part is decreasing. Consequently, as far as the intestine is concerned, regeneration usually ceases between the sixth and seventh days. After this time it is a matter of growth in the newly formed tissue itself.

Mesoderm.

The method of formation of the new mesoderm is not as generally agreed upon as is that of the new intestine. Hepke ('97) in Naids, von Wagner ('06) in *Lumbriculus* and Abel ('02) in *Tubifex* and *Nais*, among others, maintain that the new mesodermal structures originate from ectodermal cells which migrate into the cœlom. Randolph ('92) and Iwanow ('03) in different species of *Lumbriculus* and Krecker ('10 and '23) in *Tubifex* and *Limnodrilus* find that these structures are derived from relatively unmodified cells of the mesoderm to which the term neoblasts was first applied by Randolph. Krecker ('23) reports that they are ordinarily found on the posterior surfaces of the septa in a quiescent state. After a worm is cut they enlarge to 8 or 10 times their former size and then migrate along the nerve cord to the wound region. According to his data these cells are activated on the seven septa nearest the wound with those in the four nearest segments giving the greatest response. In my preparations of individuals which have been regenerating for either three or four days a number of clearly distinguishable neoblasts are always present as far as eight or nine segments from the wound. Two examples, one of a three-, the other of a four-day regenerate, are given in Table IX. All the cells which lie upon the septa and are clearly neoblasts with nucleoli of 2 μ or over are included in these counts. All are found on the posterior surfaces of the septa and are confined almost entirely to the ventral portion. These cells correspond presumably to the later stages of metamorphosis described by Krecker ('23). In both of these cases nine segments are clearly contributing neoblasts while the tenth and eleventh are ap-

parently not involved. It is a rather common occurrence to find one or two neoblasts on a septum in an uninjured worm so that the few seen in these last segments are in accordance with what is to be expected. In making these counts, not only leucocytes, as suggested by Krecker ('23), but also nephridial cells are apt to be mistaken for neoblasts in early stages of metamorphosis. Cells of this type, as clearly demonstrated by examination of adjacent sections, are shown in Fig. 9. Both of these types of cells have quite large nuclei and nucleoli and many nephridial cells are of course in contact with the septa.

TABLE IX.

NEOBLASTS.

Segments from the wound	1	2	3	4	5	6	7	8	9	10	11
3-day regenerate	17	13	16	13	9	6	7	3	6	1	1
4-day regenerate	15	19	18	14	8	7	8	8	7	2	1

On the four or five septa nearest the wound, therefore, neoblasts are most abundant, as also observed by Krecker ('23). About nine segments in all, however, seem to be involved while Krecker reports the maximum distance as seven. The region involved in the production of neoblasts is, therefore, approximately the same as that in which cell proliferation occurs in the intestine.

Ectoderm.

The ectoderm, unlike the other two types of tissue already discussed, regenerates solely from cells in the immediate vicinity of the wound. That cells from the old hypodermis form the new hypodermis and nerve cord is generally agreed upon. Krecker ('10, p. 430) describes the marked enlargement of certain ectoderm cells "not directly opposite the nerve but somewhat dorsally, between it and the central longitudinal axis of the body." These cells increase in size and become so changed that, as he expresses it (p. 434), "were these cells seen alone they would be immediately considered neoblasts." Of the changes which take place in cells other than those of this particular region, however, he makes no more than the simple statement (p. 430) that "of course the ectoderm cells are greatly enlarged elsewhere than on the ventral side."

Twelve hours after a worm is cut the wound has healed and in those ectoderm cells which are brought into a terminal position by this process the nucleoli have begun to enlarge (Fig. 10). The cells themselves are beginning to elongate somewhat and accompanying this process the nuclei are changing to the oval shape more or less typical of elongated cells. The nuclei themselves are not appreciably enlarged at this time but the nucleoli have increased from mere dots to quite evident structures about 1 μ in diameter. Cells of this type are not confined to any definite part but make up the entire terminal portion of the hypodermis. After one day of regeneration the enlargement has gone still further but there is no marked difference in size between cells in the ventral portion and those dorsally located. The average size of the nuclei at this time is 4.5 x 7.2 μ and of the nucleoli 1.4 μ.

On the second day, however, characteristic differences make their appearance (Fig. 11). The cells which are dorsal or lateral show little if any change from the preceding day. In the median ventral region just posterior to the end of the nerve cord many of the cells have grown considerably but all gradations in size can be found between the largest of these and the cells found elsewhere in the bud. In the larger cells the nucleoli are oval and about one third the dimensions of the nuclei. The average size of ten of the larger nuclei found in two adjacent sections is 6.1 x 8.4 μ, with the nucleoli of the same cells averaging 1.9 x 2.8 μ. On the following day the nuclei of the dorsal cells are of about the same size as on the second day but many of the nucleoli have enlarged to 2 μ. In the ventral region the changes are even more marked. A number of the cells have enlarged enormously and have nuclei of about 8 x 11 μ, with nucleoli averaging about 3.5 μ. It is this stage of the hypodermal cells to which Krecker applies the term "metamorphosed" ectoderm and which may be most easily confused with neoblasts. Krecker suggests that the fact that the neoblasts come to lie near such cells is probably the reason why so many workers have thought the neoblasts to be products of the ectoderm, which seems very plausible.

Krecker ('10, p. 436) suggests, as a theory of the formation of

these much enlarged cells, that "the neoblasts have a redifferentiating effect upon the cells of the ectoderm." A close examination of the facts, however, demonstrates that the cases which he cites in proof may all be explained in another way.

In the course of regeneration the enlargement of the hypodermal cells does not appear to begin suddenly soon after the arrival of neoblasts at the wound. It seems rather to be a continuous process having its inception at the time the worm is cut. A certain time is required for the cells of the hypodermis to enlarge and those on the ventral side do not change any more rapidly than those elsewhere. They do, however, show a greater response than the others with the result that their enlargement continues after the others have slowed down. In sections of twelve-hour regenerates the ectoderm is quite different from that in uninjured individuals and the process of enlargement is clearly under way. At this time neoblasts are rarely found at the wound. Krecker ('10, p. 422) says that "twelve hours after the operation . . . in one of these (individuals) there was a neoblast at the wound, but none was migrating. In the other individual . . . two were about the wound." If it is true, then, that the enlargement of the ectoderm begins before twelve hours after the cut is made, and it certainly appears to, it is improbable that the neoblasts could have been the cause. A much simpler explanation, which seems to satisfy all of the requirements of the facts at hand, is that whatever is the underlying cause of the metamorphosis of the neoblasts on the septa is likewise the cause of the enlargement of the cells of the hypodermis at the wound. Just what may cause the neoblasts to metamorphose is not discussed by Krecker.

This view that the transformation of the ectoderm cells is independent of the presence of the neoblasts is entirely in accord with the cases cited by Krecker ('10) in proof of his theory. In one individual (p. 433) "even after three days there was no enlargement of the ectoderm. No neoblasts were about the ectoderm, in fact there was only one neoblast to be seen and this was along the nerve some distance away." He says later (p. 436) that this "exception cited in which no change in the character of the ectoderm cells occurred in the absence of the

neoblasts is of considerable significance." This case may be equally well explained in accordance with the view of an independent transformation. The factor which brings about the metamorphosis of the neoblasts and ectoderm is either absent or, because of the physiological condition of this particular individual, produces no effect. It is reasonable to suppose that, if the same underlying cause brings about like changes in these two types of cells and if they are equally susceptible to activation, one type will not undergo a process of transformation when the other does not. In all other cases given by Krecker for posterior regenerates he himself states (p. 435) that "the evidence adduced has to do only with instances in which neoblasts are found about the metamorphosed ectoderm cells." Admittedly neoblasts are present in all cases where the ectoderm cells are greatly enlarged, but an explanation for this is easily found. Neoblasts migrate only along the ventral nerve cord and so they are found in the ventral region of the bud. The ectoderm cells which are greatly enlarged are those which are to give rise to the nerve cord in the ventral region. The close proximity of these two types of cells in the regenerating bud seems to be due to the fact that both bear a definite relation to the nerve cord—those of the hypodermis to form the new portion of the nerve cord in the bud and the neoblasts using it as a pathway to the wound region. In the discussion of anterior regeneration his statement that no metamorphosis of the ectoderm and no migration of the neoblasts occurs in this type of regeneration will be considered.

The development of the setigerous glands and the production of the setæ have for the most part been neglected in the work on regeneration. Bergh ('90) and Penners ('23) both give something of a description of their formation during embryonic development. In regeneration it is difficult to observe the early stages due to the great number of cells scattered throughout the growing bud. By the fifth day the cells of the glands in the two or three segments of the bud nearest the old tissue stand out clearly since at this time the other cells of these segments have become arranged in a more orderly fashion. The nuclei of the hypodermal cells near the old tissue have begun to take on the appearance typical of the older cells. They are

oval—measuring about 3.5 x 6 μ—and contain nucleoli less than 1 μ in diameter. The nuclei of these gland cells, however, have not decreased in size but are about the same as those of the enlarged ectoderm cells of the dorsal and lateral regions (Fig. 12). The average measurement of twenty-five of these is 5.2 x 7.3 μ with nucleoli 1.8 μ in diameter. Occasionally double nucleoli are to be found. The fact that these cells push into the cœlom offers another point of confusion which may have led early workers to think that the ectoderm migrated into the cœlom to form the new mesodermal structures.

It will be seen that in all three types of cells (endodermal, mesodermal and ectodermal) which take part in the formation of new tissue at the posterior end there is one outstanding feature in common. The nuclei and nucleoli enlarge. The greatest changes occur in the neoblasts and in the cells of the ventral portion of the hypodermis. In both of these types the nuclei of the fully transformed cells are eight or nine times as large as ordinarily. The nucleoli increase even more in proportion, particularly in the hypodermal cells in which they enlarge from mere dots to structures over 3 μ in diameter. Even in the gut cells where the nucleoli are usually of considerable size there is probably a ten-fold increase. The significance of these facts will be discussed later.

Origin of New Tissue in Anterior Regeneration.

Endoderm.

In anterior regeneration as in posterior regeneration the majority of investigators—Rievel ('96), Haase ('98), von Wagner ('00), Iwanow ('03) and Krecker ('10)—are agreed that most of the intestine of the regenerating bud is formed by the growth of that in the old part. This formation of new tissue involves about eleven or twelve segments here as in posterior regeneration. The nucleoli are considerably enlarged at the end of the first day and double nucleoli are fairly common. Only a few mitoses, however, are present. The numbers both of double nucleoli and of mitoses reach a maximum between the second and third days. On the fourth day both are fewer in number in the more distant segments. In the case of double nucleoli this decrease is partially

compensated by an increase in the region nearest the wound. Mitoses, however, are somewhat less frequent in this region too. As in posterior regeneration the numbers continue to decrease and by the seventh day both of these features are rare in the gut of the old part. Tables X. and XI. give records for two- and four-day regenerates, respectively. Tables XII. and XIII. give comparative data for the nearer and more distant segments for double nucleoli and mitoses, respectively.

There is little, if any difference, then, in the behavior of the cells of the intestine in anterior and in posterior regeneration.

Table X.

2 Days of Regeneration.

Segments from the wound.	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Double nucleoli.....	27	26	21	27	34	30	30	33	32	29	16	16	1	1
Mitoses.....	12	6	3	7	8	4	3	4	2	3	2	0	1	0

Table XI.

4 Days of Regeneration.

Segments from the wound.	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Double nucleoli.....	38	31	29	32	34	26	14	7	8	6	2	3	3	1
Mitoses.....	12	4	3	4	7	3	1	0	1	1	0	0	1	0

Table XII.

Double Nucleoli.

Days of Regeneration.	Segments from Wound.		Total.
	1–5 (incl.).	6–12 (incl.).	
2	135	186	321
4	164	66	230

Table XIII.

Mitoses.

Days of Regeneration.	Segments from Wound.		Total.
	1–5 (incl.).	6–12 (incl.).	
2	36	18	54
4	30	7	37

Mesoderm.

Many investigators—Hepke ('97), von Wagner ('00) and Abel ('02) among others—are of the opinion that the mesoderm both of anterior and of posterior regenerates is formed from the ectoderm. The weight of evidence in posterior regeneration, as previously mentioned, now seems to favor mesoderm formation from neoblasts. In anterior regeneration Iwanow ('03) in *Lumbriculus variegatus* and Krecker ('10) in *Tubifex* and *Limnodrilus* find that the new mesoderm is formed from the old mesoderm and not from regeneration cells as in posterior regeneration. They find a distinct fraying out of the longitudinal muscles at the wound. Many of the cells then lose their contractile substance and wander about in the cœlom where they become mixed with other cells—peritoneal, connective tissue and ectodermal—so that it is "hard to distinguish between the various types" (Krecker, '10, p. 441). After about the fifth day of regeneration they begin to develop contractile substance and to become arranged in muscle masses. The production of the body musculature is much slower in anterior than in posterior regeneration. "In specimens killed three weeks after the operation the musculature of this region was still in a very undeveloped state" (Krecker, '10, p. 442).

As the matter stands at present, then, investigators are divided into two groups regarding the formation of the new mesoderm. Those in one believe that it is derived from the ectoderm, those in the other that it is produced by a partial dedifferentiation followed by a redifferentiation of the cells of the old mesodermal structures. Those who support an ectodermal origin of the mesodermal structures believe that the mesoderm is formed in the same manner at both ends. Those of the other group, however, think that the origin is due to one type of mesodermal cell at the posterior end—the neoblasts—and to another at the anterior end—the specialized cells which dedifferentiate.

In anterior regeneration, just as in posterior regeneration, there are many opportunities for confusion. In the former it is even more difficult than in the latter to follow just what happens. The migration of ectoderm cells into the cœlom in the formation of the cerebral ganglion might easily mislead one. In addition

to this the presence of many other cells of similar appearance make it practically impossible to say, from an examination of fixed specimens, just what cells do form the various tissues. Since in posterior regeneration it is quite apparent that ectoderm is not involved in the formation of the septa or longitudinal muscles it seems reasonable to assume that it does not take part here. This view is supported further by the fact that cells of mesodermal origin are present in the bud.

Regarding the conception that there is a partial dedifferentiation of the old muscle cells which then form the longitudinal musculature of the bud, there seems to be conflicting evidence. These cells certainly do fray out and some of them seem to lose their contractile substance in the segment injured by the cut. Such a behavior is not, however, peculiar to the anterior end in *Lumbriculus inconstans*. Just as other cells are affected by the cut so many muscle cells are dislodged or injured. It is not improbable that such cells should then lose their contractile substance due to the injury; such a change might, therefore, be a step on the road to destruction rather than on that to repair. The fact that this same sort of change does take place at a posterior cut-surface, where the muscle cells are not involved in the regenerative processes, seems to support this view. The migrating spindle-shaped cells, which appear between the first and second days in *Lumbriculus*, are clearly derived from the hypodermis (Fig. 13). These cells migrate into the cœlom and there form the cerebral ganglion. It may perhaps be this type of cell which has been observed by Krecker in *Tubifex* and *Limnodrilus*.

Further evidence is derived from a study of neoblasts in anterior regenerates. Krecker finds that in the species with which he worked they are usually not activated at all posterior to a cut and that resting neoblasts may be found on a nearby septum. "The individuals upon which these observations were made were all killed three weeks or more after the operation so that the failure of the neoblasts to act as they do at the posterior end could hardly have been due to lack of time" (Krecker, '10, p. 437). In the individuals used in his experiments—of the genera *Tubifex* and *Limnodrilus*—anterior regeneration does not

take place at the level of the twentieth somite where these cuts were made. It does not seem strange, therefore, that at the end of three weeks all neoblasts should be resting even if activation had taken place three weeks previous. In *Lumbriculus* anterior regeneration does take place at all except very posterior levels. The amount of tissue is, of course, limited to a few segments, usually five or six, rarely seven (von Wagner, '00).

Counts made of the number of clearly recognizable neoblasts (intermediate and definitive stages of Krecker, '23) present on the various septa in three- and four-day regenerates are given in Table XIV. These cells all possess nucleoli of approximately 2 μ or over (Fig. 14).

TABLE XIV.

NEOBLASTS.

Segment from the wound	1	2	3	4	5	6	7	8	9	10	11
3-day regenerate	6	21	14	9	11	13	3	3	4	2	1
4-day regenerate	16	14	23	13	7	8	2	6	3	1	1

These figures do not differ greatly from those for posterior regenerates (Table IX.). The small number of neoblasts present on the first septum of the three-day regenerate may be explained in part by the fact that this septum was partially torn away by the cut in this particular case. About four segments seem to produce neoblasts more abundantly than the rest and about nine segments in all are apparently involved. The more active region extends farther, however, in the three-day regenerate; the inclusion of the first septum in the cut brings this about. The neoblasts, then, are activated to about the same extent as at the posterior end. The number present in the bud at the end of two days, however, indicates that either not as many migrate anteriorly or else the migration is much slower and the cells are used up as they get to the wound. No mass of large cells is to be found in the ventral part but several cells are usually present and a number can generally be found migrating along the nerve cord (Fig. 15). The apparently slow migration of the neoblasts anteriorly is perhaps the reason why the mesoderm in the newly formed head does not show signs of development until after the

fifth day (Krecker, '10). After the third day it is difficult to say just what does happen in the bud region. Many metamorphosed ectoderm cells are present and things are so confused that it is difficult to distinguish the various types of cells, particularly those as similar in appearance as are the neoblasts and the much enlarged cells from the ventral hypodermis.

The process of formation of new septa and longitudinal muscles by cells known to be derived from neoblasts has not been observed. The fact that neoblasts metamorphose in considerable numbers and are frequently seen migrating along the nerve cord is, nevertheless, evidence which seems to indicate that they play some part in anterior regeneration in *Lumbriculus*. If such is the case, the mesodermal structures are formed in the same manner in both types of regeneration, just as are the ectoderm and endoderm.

Ectoderm.

As in posterior regeneration only those cells in the immediate vicinity of the wound take part in the formation of the new ectodermal structures. There occurs a similar enlargement of all these cells during the first day or so, no one region undergoing any more extensive change than any other. Iwanow ('03) in his discussion of the formation of the new nerve elements at the anterior end describes a marked enlargement of the ectoderm cells. Krecker ('10, p. 433) says that the ectoderm cells "undergo no such metamorphosis" as at the posterior end, explaining this on a basis that no neoblasts migrate anteriorly to stimulate it to activity. As in the case of posterior regeneration, however, by the second day there are cells on the ventral side which have quite large nuclei and nucleoli, and by the third day they have reached a considerable size (Fig. 16). The area over which this marked enlargement extends is greater than in posterior regeneration. Large nuclei are present in cells found over a considerable portion of the ventral and ventro-lateral hypodermis and even somewhat dorsally in the terminal portion. The largest cells, nevertheless, are confined for the most part to the ectoderm in the vicinity of the mid-ventral line. This region extends from the end of the old nerve cord anteriorly. The nuclei and nucleoli measure about the same as in the transformed cells of the

regenerating tail (8 x 11 μ and 3.5 μ, respectively). In the dorsal portion of the terminal hypodermis many of the cells are apparently in the process of breaking away from the epithelium to migrate into the cœlom where they form the cerebral ganglion (Fig. 13). They become elongated and slender in this process and develop an appearance which answers well the description of cells which Krecker ('10, p. 441) believes are mesoderm cells migrating anteriorly to form the new longitudinal muscles—"spindle-shaped cells with slightly granular cytoplasm and large nucleus containing a deeply staining nucleolus."

Regarding the activation of the ectoderm by the presence of the neoblasts, there is no further evidence from anterior regeneration. Neoblasts apparently migrate anteriorly and the ectoderm is also transformed, similar processes to those occurring at the posterior end.

From these observations it is apparent that not only the ectodermal and endodermal elements of the regenerated head are derived in the same manner as at the posterior end but the mesodermal structures as well. Just as in posterior regeneration the neoblasts apparently metamorphose and migrate to the wound region and the cells of the ventral portion of the hypodermis become greatly enlarged. About ten or eleven segments seem to be involved in the regenerative processes except in the case of the ectoderm in which the changes are confined to the immediate vicinity of the wound as in posterior regeneration.

Discussion.

The production of new tissue in the anterior regeneration of microdrilous annelids seems to be essentially the same as in the posterior regeneration in these forms. Iwanow ('03) in *Lumbriculus variegatus* and Krecker ('10) in *Tubifex* and *Limnodrilus* describe the formation of the new mesodermal tissue from neoblasts at the posterior end but believe that there is a dedifferentiation of old mesoderm to form the new at the anterior end. The loss of contractile substance by some of the longitudinal muscle cells at the cut surface, however, occurs at both ends. The spindle-shaped cells abundant in the dorsal part of the bud in anterior regeneration may be seen in *Lumbriculus inconstans*

to be derived from the hypodermis, migrating into the cœlom to form the cerebral ganglion (p. 295). The fact that many neoblasts metamorphose and migrate anteriorly is evidence that they probably take part in the building of the new mesoderm in anterior as well as in posterior regeneration. The presence in the bud of other cells of similar appearance makes it practically impossible to follow the laying down of these structures from cells of known origin. It has for some time been agreed by investigators that both endodermal and ectodermal structures are formed in the same manner in both types of regeneration, that is, from the old intestine and body wall, respectively. It seems evident then that the mesodermal structures are produced in the same way at both ends and are not an exception as previously believed.

An explanation for the formation of the new mesoderm from more or less undifferentiated mesodermal cells rather than from the muscles may perhaps be found in the fact that the cytoplasm of the muscle cells is highly modified. While the cells of the hypodermis and intestine are of a simple, cuboidal or columnar shape, the development of the contractile substance by the muscle cells brings about an extensive modification of the cytoplasm of these cells. Consequently, cells from the peritoneum, less modified than the others are called upon to form the tissue in the regenerating bud. The development of the new nerve cord is a process not essentially different. In this case the cytoplasm is considerably modified in the formation of fibers and, instead of the nerve cord near the wound dedifferentiating to produce the new tissue, the hypodermal cells of the ventral side are called upon to furnish the new material. While these facts may not necessarily indicate that one type of cell is any "more differentiated" than another, the muscle and nerve cells in *Lumbriculus* certainly are less susceptible to the activating stimulus than are those of the other tissues.

In regeneration the ectoderm cells enlarge considerably especially those in the median ventral line which may develop nuclei as large as $8 \times 11\ \mu$ with nucleoli between 3 and $4\ \mu$ in diameter. Krecker ('10 and '23) is of the opinion that this change in the ventral cells is due to "some influence" of the

neoblasts. Evidence presented here (pp. 290 and 297) does not support this idea but rather the view that a similar underlying cause brings about the changes which occur in the metamorphosis both of the neoblasts and of the ectoderm cells. During the first two days of regeneration all the cells of the hypodermis in the immediate vicinity of the wound develop large nuclei and nucleoli. After this time the ones found dorsally and laterally slow down whereas those in the ventral region continue to enlarge. Neoblasts are rarely, if ever, found at the wound at the time when the increase in size of the hypodermal cells becomes apparent (between six and twelve hours after the cut). From that time until the largest cells are formed the enlargement seems to be a continuous process without any sudden change or increase in rate which might be produced by the presence of neoblasts. The fact that in posterior regeneration metamorphosed ectoderm cells and neoblasts are always found very near one another is cited by Krecker ('10) as proof that there is some relation between them. This proximity of these two types of cells is perhaps equally well explained if the reason for the presence of each in the ventral region is considered. The cells of the ventral hypodermis are transformed in the process of proliferation and migration to form the nerve cord of the regenerating region. The neoblasts migrate to the posterior end from the old tissue along this same structure. These cells, then, are brought together not because one causes the modification of the other, but rather from the fact that each type has a certain relation to the nerve cord. The view that the same cause produces the changes in the cells of both types seems to fulfill all the requirements and to be a simpler explanation than that given by Krecker. Furthermore, there is no apparent reason why the ectoderm, any more than the endoderm, should need to be activated by another type of cell.

Function of the Nucleolus.

Various theories have been advanced regarding the function of the true nucleolus or plasmosome. Montgomery ('98) and Ludford ('22) give a rather complete discussion of the work of many investigators. The more recent results seem to indicate

that the nucleolus bears some relation to the activity of the cell body and nucleus. Many cases have been reported of nucleolar extrusions preceding the formation of yolk granules. Ludford ('22) also reports that in the more active cells of the endoderm of *Limnæa* the nucleoli are larger than in those of the hypodermis. He is "inclined, therefore, to regard the size of the nucleolus as an indication of the degree of metabolism existing in the cells—the greater the metabolic activity, the larger the total volume of nuclear (nucleolar ?) matter present in the nucleus, or extruded into the cytoplasm" (p. 139). Wilson ('25, p. 96) also believes that there is a "question whether the nucleolus may not play a more active and important part in cell metabolism than most writers have hitherto assumed."

An examination of slides of *Lumbriculus*, both of uninjured and of regenerating individuals reveals no cases of nucleoli which could be interpreted as being extruded into the cytoplasm from the nucleus. There is considerable evidence, however, that nucleolar size is in some manner an indication of the degree of metabolic activity of the cell.

In uninjured worms the gut cells of the first eleven or twelve segments have small nucleoli. Similarly those for some distance from the anal opening have a comparatively small amount of nucleolar substance. In the intermediate portion, however, relatively large nucleoli are present. The cells of the mouth cavity and esophagus naturally do not take as great a part in the digestive processes as do those found more posteriorly. In the mid-gut the digestive fluids are being poured into the lumen and the food is being absorbed. Consequently considerable cell activity is necessary. Toward the posterior end such activity naturally drops off again. The size of the nucleoli, therefore, parallels more or less the extent of the activity expected of the cells in the various regions of the gut (p. 281).

Furthermore, in the case of the setigerous glands, the nucleoli of the cells are large in the growing tail region and in a regenerating bud where the new setæ are being rapidly formed (pp. 281 and 291). In the old segments of a worm, however, where the setæ have been present for a considerable time, the nucleoli are usually small (p. 281). The cells in the active

portion of the nephridia possess nucleoli of considerable size, too. In fact, these cells are very similar in appearance to intermediate neoblasts, from which they may be distinguished by the presence of large granules in their cytoplasm (p. 288). Similarly, as described by Krecker ('23), the phagocytes have very large nucleoli.

Again, in the growing tail, just as in the regenerating individual, the hypodermal cells have enlarged nucleoli, particularly on the ventral side where the nerve cord is being formed (p. 280). Those cells which are forming the new mesoderm—presumably derived from the primary mesoblasts in the embryonic development (Wilson, '89 and '92)—also have very large nucleoli, differing very little in appearance from the neoblasts in a regenerating tail (Randolph, '92). In contrast to this, in the older part of the worm, the nucleoli of the mesoderm and ectoderm cells are very small, in the case of the latter mere dots under a magnification of 1,000 ×.

In regeneration, as already mentioned, the nucleoli of the neoblasts and hypodermal cells become greatly enlarged. There is also at this time an increase in the amount of nucleolar substance in the gut cells. For ten or eleven segments from the wound, the nucleoli enlarge, a process followed by the appearance of numerous instances of double nucleoli—two within a single nucleus (p. 285).

This occurrence of double nucleoli is taken by Iwanow ('03) and Krecker ('10) as evidence that amitosis is frequent in the production of the new gut tissue. In *Lumbriculus* there is no evidence of any division or even of a clearly defined constriction in any of the nuclei of the gut which contain two nucleoli. The individual nucleoli in the case of the double one are usually smaller, and in no case larger, than those in the neighboring cells where but a single nucleolus is present. It seems, then, that the division of the nucleolar material into two parts is not in preparation for a succeeding cell division. Rather as this material accumulates it continues to exist in a single droplet until it reaches a certain size and then divides. This splitting into two parts may perhaps be due to the fact that a droplet of material of its consistency and composition has a certain

maximum size beyond which it cannot exist as an individual droplet under the conditions existing in the nucleus. This view is in accord with our knowledge of the limitations to drop size in emulsions.

After one day of regeneration many cells show this increase in the amount of nucleolar material, as evidenced by the occurrence of a number of double nucleoli as well as the enlargement of the single ones. On the second and third days the frequency of double nucleoli reaches a maximum and after that time falls off slowly. Following the increase in nucleolar material, there appear numerous cases of mitosis. It seems probable that this increase in nucleolar substance is indicative of a heightened activity on the part of the cells in preparation for cell division.

A comparison of the amount of nucleolar material in the various types of cells taking part in regeneration reveals a distinct parallelism between this amount and the relative activities of these cells. There are four general types of cells involved: (1) those of the old gut which form the new gut; (2) those of the old dorsal and lateral hypodermis which build the new hypodermis; (3) those of the old ventral hypodermis of which the special function is to furnish the material for the nerve cord in the regenerating bud; finally (4) the neoblasts which form the new mesodermal structures. Of these, the cells of the first two types maintain to a certain extent their usual epithelial arrangement, only a comparatively small amount of migratory activity occurring. Their nucleoli enlarge considerably but by no means as much as in the case of the other two types. The ventral ectoderm cells and the neoblasts, when fully transformed, are of about the same general size and appearance and have nucleoli of nearly twice the diameter of those in the other cells. The ventral ectoderm cells must naturally undergo rapid proliferation to supply all of the material necessary for the nerve cord; the neoblasts must migrate to the wound region and there multiply with considerable rapidity. There is some relation in *Lumbriculus*, then, between the functional activity of the cells and the amount of nucleolar material present in them. This is a conclusion similar to that drawn by Ludford ('22) from a study of *Limnæa* with particular reference to the behavior of the nucleolus in oögenesis and cleavage.

Summary.

Nuclei and Nucleoli in Uninjured Individuals.

1. The nuclei and nucleoli of the hypodermal cells are small except in the growing tail region. Here they are enlarged, especially in the cells on the ventral side which are involved in the formation of the new nerve cord.

2. Large nucleoli are present in the cells of the setigerous glands near the growing region of the tail. In old segments they are small.

3. The gut nucleoli are small in the first twelve segments. They are larger from this region up to twenty or thirty segments from the posterior end. In these segments, they are again small.

4. Double nucleoli are occasionally found in the mid-gut, where large nucleoli are present.

Origin of New Tissue in Regeneration.

5. Double nucleoli and mitoses are found in the intestine for eleven or twelve segments from the wound. In this same region the nucleoli are considerably enlarged.

6. Cell proliferation in the old intestine practically ceases between the sixth and seventh days of regeneration.

7. Neoblasts metamorphose and migrate to the wound at the anterior end as well as at the posterior end. At least eight or nine segments furnish these cells, the four or five nearest the wound apparently playing the most important part as observed by Krecker.

8. The failure of the muscle and nerve cells of the old part to form the corresponding new structures in regeneration is perhaps due to the fact that the cytoplasm of these cells has become highly modified, thus rendering them less susceptible to activation.

9. The spindle-shaped cells in the dorsal portion of the bud cavity at the anterior end are derived from the hypodermis and not from the muscles of the old part.

10. In both anterior and posterior regeneration the nuclei and nucleoli increase in size in the ectoderm cells in the immediate

vicinity of the wound. This enlargement is no more rapid in one part than in another; it continues longer in the ventral cells so that by the second day it is greater there.

11. The metamorphosis of the ectoderm is not in all probability due to the proximity of the neoblasts, as supposed by Krecker, but instead to an independent transformation.

12. The cells of the setigerous glands in the new bud have large nuclei and nucleoli.

13. A feature common to all the cells which take part in the formation of the tissues in the regenerating bud is the presence of large nuclei and nucleoli.

14. The amount of nucleolar material present in a cell seems to be an index of the activity of its nucleus both in cell-metabolism and in preparation for cell division.

15. In *Lumbriculus* there is no evidence of any division or even of a clearly defined constriction in any of the nuclei of the gut which contain two nucleoli.

16. The presence of two nucleoli in a single nucleus is not a step in amitosis, as many have supposed, but is due to the increase in nucleolar substance beyond the amount which can exist within that particular nucleus as a single droplet.

17. The various tissues seem to be derived in the same manner both in anterior and in posterior regeneration.

Conclusions.

1. Both in anterior and in posterior regeneration the mesoderm is formed from neoblasts. Iwanow and Krecker are in error in the belief that cells from old specialized mesodermal structures form the new ones in the anterior regeneration of *Lumbriculus*.

2. There is a certain predetermined area of the hypodermis on the ventral side which metamorphoses preparatory to the formation of new nervous tissue during regeneration. The cells of this region are probably activated by the same stimulus as are the neoblasts. Krecker's view that the neoblasts have an inciting effect on the cells of this region seems unfounded.

3. The amount of nucleolar material present in a cell seems to be an index of the activity of its nucleus both in cell-metabolism and in preparation for cell division. Two nucleoli within a single

nucleus are the result of an increase in nucleolar substance beyond the amount which can exist within the nucleus as a single droplet. Their presence is not a step in amitosis.

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KEY TO PLATES.

All figures are photomicrographs. With the exception of Fig. 11, all were taken with a Zeiss apochr. 2 mm., eyepiece 10; these are magnified 640 ×. For Fig. 11, a B. and L. apochr. 4 mm. and eyepiece 6 were used; this is magnified 290 ×. The following are the symbols used in the figures:

a., anal opening,
c., cuticle,
chl., chloragogue cells,
d. e., enlarged dorsal ectoderm cells,
d. n., double nucleoli,
g., gut,
gl., gland,
h., hypodermis,
m., mitosis,
m. c., mouth cavity,
m. e., metamorphosed ectoderm cells,
mes., mesoblast,
migr., migrating ectoderm cell,
n., nucleus containing nucleolus,
neph., nephridial cell,
nb., neoblast,
nv., nerve cord,
s., seta,
sep., septum.

Plate I.

Uninjured Individuals.

Fig. 1. Mouth cavity and hypodermis, showing small nuclei and nucleoli in this region.

Fig. 2. Growing region at posterior end.

Fig. 3. Setigerous gland of an old segment.

Fig. 4. Gut of fourth segment.

Fig. 5. Gut of thirteenth segment.

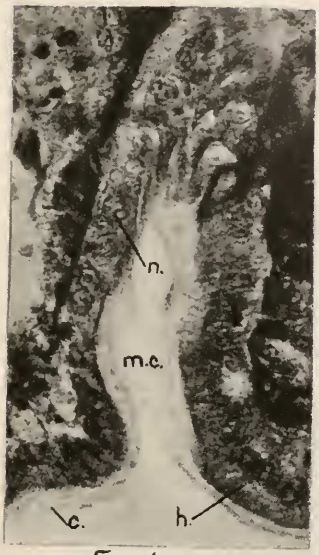

Fig. 1.

Fig. 5.

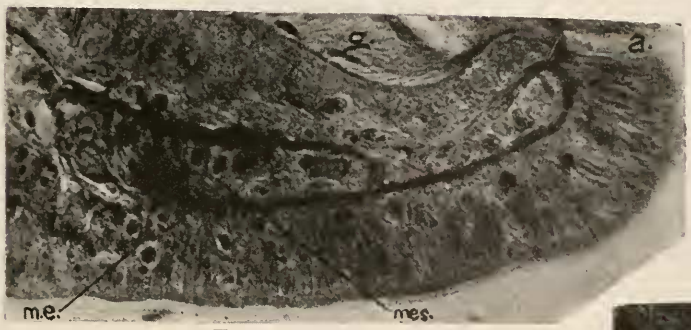

Fig. 2.

Fig. 3.

Fig. 4.

Plate II.

Regenerates.

Fig. 6. Two-day posterior regenerate. Gut cells of second segment from wound.

Fig. 7. Same individual as in Fig. 6. Gut cells of tenth segment from wound.

Fig. 8. Same individual as in Fig. 6. Double nucleoli in gut cells of fourth segment from the wound.

Fig. 9. Three-day anterior regenerate. Nephridial cells in a position in which they might be mistaken for neoblasts. Arrow points toward the anterior end.

Fig. 10. Twelve-hour posterior regenerate. Enlarging ectodermal cells in wound region.

Fig. 11. Posterior regenerating bud at the end of two days.

Fig. 6

Fig. 7.

Fig. 10.

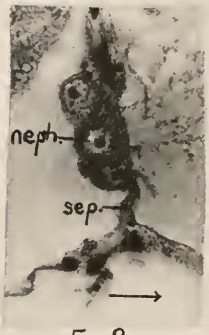

Fig. 9.

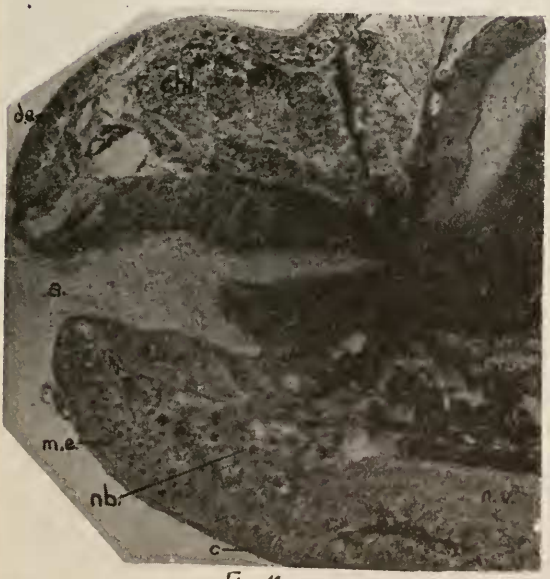

Fig. 11.

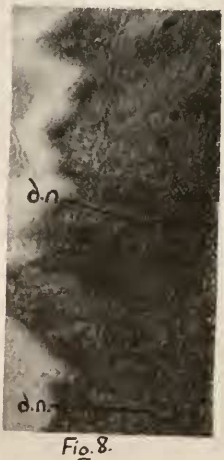

Fig. 8.

Plate III.

Regenerates.

Fig. 12. Six-day posterior regenerate. Setigerous glands producing new setæ.

Fig. 13. Two-day anterior regenerate. Spindle-shaped cells of ectoderm migrating into bud cavity.

Fig. 14. Two-day anterior regenerate. Neoblasts metamorphosing on the posterior surface of the fifth septum from the wound. Arrow points toward anterior end.

Fig. 15. Two-day anterior regenerate. Neoblasts at anterior end of nerve cord.

Fig. 16. Three-day anterior regenerate. Metamorphosed cells of ventral ectoderm.

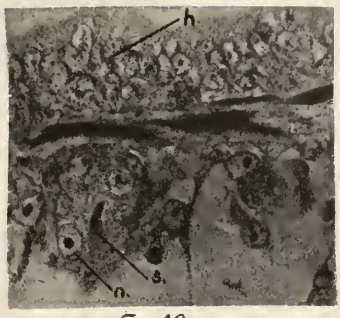

Fig. 12.

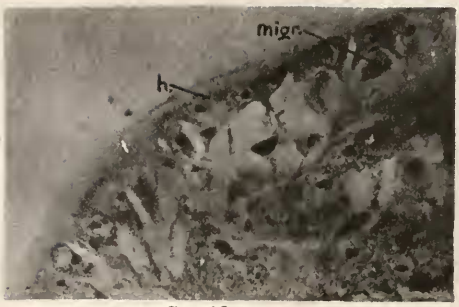

Fig. 13.

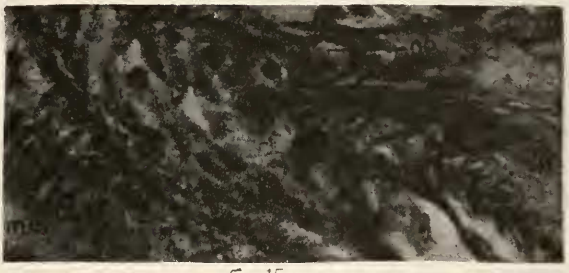

Fig. 15.

Fig. 14.

Fig. 16.

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BIOLOGICAL BULLETIN

THE EFFECT OF CYANIDES ON THE SWELLING OF PROTOPLASM.

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Physiologists have long since attempted to formulate a theory for the cause of anæsthesia, and the results thereof have been many and varied. The exceptions to any one theory are too numerous and too important to accept any one of them as the correct theory. The final and real solution of so complex a physiological process will probably be one which will take into consideration portions of all theories now existing, or one which will introduce an entirely new factor or factors. Accounts of the various existing theories are set forth in a number of papers among which are the following: Overton ('01), Meyer ('99), Traube ('19), Warburg ('14), Mathews ('14), and Lillie ('18). This paper will make no attempt to give any evidence in support of any one of the theories, but will rather present the results obtained in using HCN and KCN as anæsthetics.

The work here reported deals with the effects of HCN and KCN on the permeability of unfertilized *Arbacia* eggs to water. An increase in the volume of the eggs, when placed in a solution, is taken as showing an increase in the permeability of the egg. Heilbrunn ('25), however, objects to calling an increase in the volume of the egg an increase in permeability. He suggests that this increase in size may be due to a decrease in surface tension; an increase in the fluidity of the interior or an increase in the extensibility of the plasma membrane. Since *Arbacia* eggs are nearly all spherical, changes in their volume may be measured by

measuring the changes in the diameters of the eggs with an ocular micrometer. Eggs from a single individual were placed in concentrations of HCN varying from $N/300$ to $N/2{,}000$. At the end of ten minutes and again after 25 and 66 minutes, eggs were pipetted into a 50 per cent. solution of sea-water (hypotonic) and their diameters measured at regular time intervals. A control was kept in which sea-water replaced the HCN solutions.

The HCN solutions were prepared by drawing over, by means of an aspirator, into a wash bottle containing distilled water, the volatile HCN gas from another bottle containing KCN to which a few drops of acid had been added. The amount of HCN going into solution was determined by titrating with 0.1 N $AgNO_3$; 1.0 cc. of the nitrate being equal to 0.013 gram HCN. It was necessary to use distilled water because the chlorides in the sea-water interfered with the titration. The desired concentrations of HCN were then made up with sea-water. All the sea-water mentioned in these experiments was sea-water from which CO_2, in excess of that in equilibrium with the CO_2 in the air, had been removed. This was done by adding 2.4 cc. of 0.1 N HCl per liter of sea-water and aerating for 18 to 24 hours. NaOH or HCl was then added to bring it to the pH of normal sea-water.

The eggs, after exposure to the cyanide solutions, were placed in syracuse watch-glasses containing the hypotonic solution and a 4 mm. objective used as a water immersion with a 10× ocular. This gave a magnification of 450×. Readings were taken one minute after placing in the hypotonic solution and thereafter every minute for ten or fifteen minutes. A stop watch was used to read time intervals. The following abstract taken from daily notes will show the routine followed throughout the experiments.

7/20/26. Temperature of room 21° C. pH of HCN solution measured colorimetrically =7.2. *9A.M.* unfertilized *Arbacia* eggs placed in 30 cc. of $N/300$ HCN in a finger bowl and covered. *9.10 A.M.* eggs pipetted into 5 cc. hypotonic sea-water in watch glass and diameter of eggs measured every minute for ten minutes. Three perfectly spherical eggs were measured each time and the average taken. *9.25 A.M.* some eggs from original $N/300$ HCN solution placed in hypotonic solution and ten one-minute readings again taken. Temperature of room 21.2° C. *10.06 A.M.*, after

exposure of 66 minutes to HCN solution, ten one minute readings again taken. Temperature of room 21.7° C.

The 10, 25 and 66 minute readings were repeated using solutions of HCN up to *N*/2,000. Similar readings were also taken using sea-water instead of HCN solutions. These served as controls. Each time that a new sea-urchin was used, the diameters of the eggs were first measured to be sure that they were approximately the same size as eggs which had been previously used.

As previously stated, the HCN was drawn over into distilled water and then diluted with sea-water. The original HCN solution (with distilled water) usually reached its saturation point as an *N*/130 HCN solution. In order to bring this to an *N*/300 HCN, an amount of sea-water almost equal to the original amount of distilled water had to be added. This in itself, therefore, was a 57 per cent. solution of sea-water and the change in volume of the eggs might very well be due to that hypotonicity instead of to the effect of the HCN. This was checked by adding to the control solutions of sea-water, as much distilled water as was contained in the various concentrations of HCN. That is, the control for the *N*/300 HCN was a 57 per cent. sea-water solution; for the *N*/500 HCN a 74 per cent. sea-water and for the *N*/2,000 HCN a 93 per cent. sea-water solution. The results with these various controls showed that only in the high dilutions, *i.e.*, 57 per cent., 67 per cent. and 74 per cent. sea-water solutions, did this hypotonicity have any appreciable effect on the volume of the eggs, but even this increase was much less than the increase in volume of the eggs previously exposed to the HCN solution. The dotted curve in Fig. 1 marked Control A is the control in 100 per cent. sea-water, while the curve marked Control B is the control in 67 per cent sea-water (comparable to the *N*/400 HCN). The controls for the other concentrations have been omitted to avoid confusion, but in every case the volumes of the eggs in the controls were less than the volumes in the respective HCN solutions. In other words, the volumes of the eggs in the HCN solutions as shown in Fig. 1 are slightly greater than they would be if the HCN solutions had been entirely made up with sea-water. Since the KCN solutions were made up with sea-water only, these precautions were unnecessary for that series of experiments.

In order to test the narcotic properties of the cyanides, eggs were inseminated and after three minutes, placed in progressive dilutions of KCN and HCN. An $N/100{,}000$ HCN solution still inhibited cell division, while an $N/30{,}000$ KCN solution was the lowest concentration which would inhibit cell division. Eggs which had been inseminated and then narcotized, were replaced in sea-water and the time for first cell cleavage to appear noted. Eggs which had been exposed to various concentrations of both HCN and KCN for varying lengths of time, were then washed by letting them fall to the bottom of a test tube filled with sea-water and then transferred to sea-water in a watch-glass. These were then inseminated to see whether the eggs were still alive after the effects of the cyanides and the hypotonic sea-water.

The effect of KCN on the permeability was followed merely for comparison with the HCN and ten one-minute readings were taken of eggs in 50 per cent sea-water which had previously been exposed for 25 minutes to concentrations of KCN varying from $N/300$ to $N/900$. The KCN solutions were made up entirely with sea-water.

Results.

Exposure of *Arbacia* eggs to HCN causes an increase in the volume of the eggs when placed in hypotonic sea-water, above that of the controls exposed to sea-water. The volume of the eggs varied directly as the concentration of the HCN and as the time of exposure to the HCN solutions. As previously stated, the fact that the HCN solutions were in themselves hypotonic in varying degrees, did not interfere greatly with the final results, since the increase in volume due to this hypotonicity was only slight as compared to the increase in volume due to the HCN. All eggs exposed to the HCN and to the sea-water controls finally reached the same equilibrium point, $4{,}518 \times 10^2\ \mu^3$. Only eggs which had approximately the same size at the beginning of the experiments, $2{,}381 \times 10^2\ \mu^3$, were used. The effect of the HCN was to hasten reaching the equilibrium point. Fig. 1, in which the volumes of the eggs after definite exposures to HCN are plotted against the time in the hypotonic sea-water, shows the rate at which the increase in volume occurs. Fig. 2 shows the

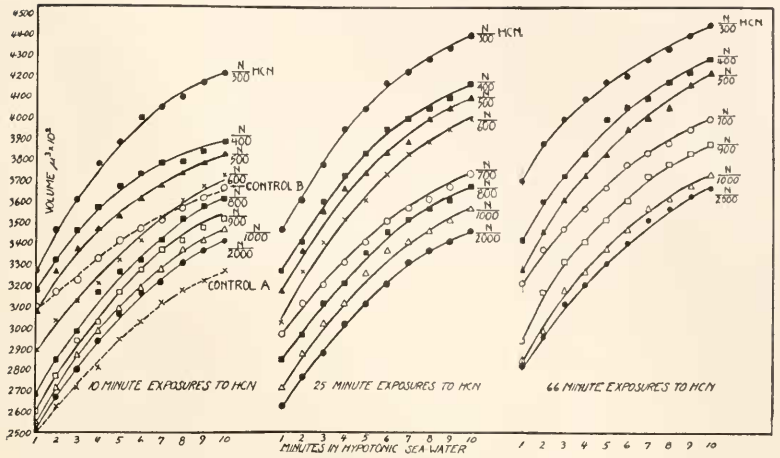

FIG. 1. Volume of eggs after definite exposures to various concentrations of HCN plotted against the time in 50 per cent. sea-water, showing the rate of increase in volume. Control A was exposed to 100 per cent. sea-water instead of HCN while control B was exposed to 67 per cent. sea-water, comparable to the hypotonicity of the *N*/400 HCN. Controls in other percentages of sea-water comparable to the remaining concentrations of HCN have been omitted to avoid confusion, but in all cases, the volumes of the eggs in sea-water were less than the volumes in the HCN solutions.

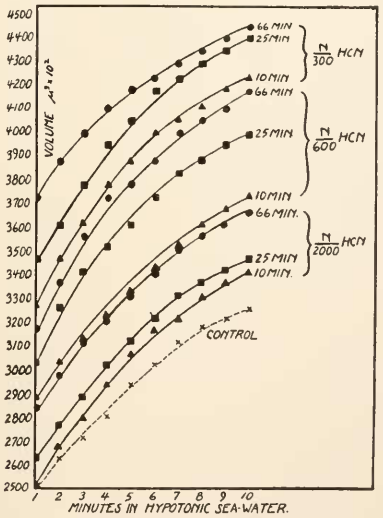

FIG. 2. Illustrating the rate of increase in volume of eggs exposed to the same concentrations of HCN but for varying time intervals. The control was exposed to 100 per cent. sea-water.

rate of increase in volume of eggs exposed to the same concentrations of HCN but for varying lengths of time. Volume here is also plotted against time in hypotonic sea-water. KCN, on the other hand, caused a decrease in the volume of the eggs, when placed in hypotonic sea-water, below that of the control exposed to sea-water. The volume of the eggs varied inversely as the concentrations of KCN. Fig. 3 shows the rate at which the volumes of the eggs decrease with increase in concentration of KCN.

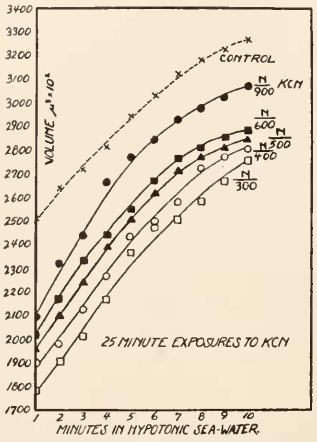

FIG. 3. Volumes of eggs after exposure to KCN showing the rate of decrease in volumes with increase in concentration of KCN. Control was exposed to 100 per cent. sea-water.

The pH of the HCN solutions varied only slightly, being 7.2 to 7.4, while the pH of the KCN solutions remained at 7.4. The work of Lucke and McCutcheon ('26*a*) shows that the volume of *Arbacia* eggs is independent of the pH of the solution except in cases where the pH is so high or so low as to injure or kill the eggs. According to the same authors ('26*b*) temperature does have an effect on the volume of the eggs in hypotonic sea-water. Although no attempt was made to keep the temperature constant during the experiments, an accurate record, taken every half hour during the experiments, was kept of the temperature of the laboratory. Conditions at Woods Hole are such that the temperature rarely varied $\pm 2°$ from 22° C.

Eggs which had been exposed to *N*/300 HCN and *N*/300 KCN for 60 minutes, by which time they had reached their maximum

or minimum volumes respectively, were returned to sea-water and fertilized. Cell division took place, showing that the eggs were alive at their greatest expansion or contraction. One cannot very well tell whether an anæsthetized egg can be fertilized while in that condition, since a normal egg does not show first cleavage until about 60 minutes after insemination. Within these 60 minutes, the narcotized condition may have been reversed, and the dividing egg be, not a narcotized egg, but a normal one. The anæsthetized egg, however, did form a fertilization membrane immediately after insemination, and in view of the statement which follows, might indicate that a narcotized egg can be fertilized, but cleavage is delayed until the narcotic has diffused out of the egg, or until the narcotized condition has been reversed. Untreated eggs normally showed first cleavage about 60 minutes after insemination. Eggs which had been anæsthetized with varying concentrations of HCN 3 minutes after insemination and then transferred to sea water, showed first cleavage at varying times always longer than the untreated egg; however, the higher the concentration of HCN used to anæsthetize them, the longer it took for first cell cleavage to appear.

Lillie ('16) has suggested a modification of the equation followed by unimolecular reactions $dx/dt = k(a - x)$, in dealing with rates of osmotic pressure in egg cell, of the form $kt = Ln\frac{V_{eq} - V_0}{V_{eq} - V_t}$ where V_{eq} is volume at equilibrium; V_0 is volume at the first instant (in sea-water); and V_t is the volume at time t. Lillie found that this equation represents the rate of swelling of fertilized and unfertilized *Arbacia* eggs in hypotonic sea-water and Lucke and McCutcheon ('26) found that it applied also to the rate of swelling in sea-water of varying hypotonicity.

That this same equation holds good in the series of experiments described in this paper, can be seen from Fig. 4. When $\log \frac{V_{eq} - V_0}{V_{eq} - V_t}$ is plotted against time in hypotonic sea-water, a straight line should result. This has been found to be the case. The values of k, the velocity constant, are given by the slope of the line. This figure shows that the higher the concentration of

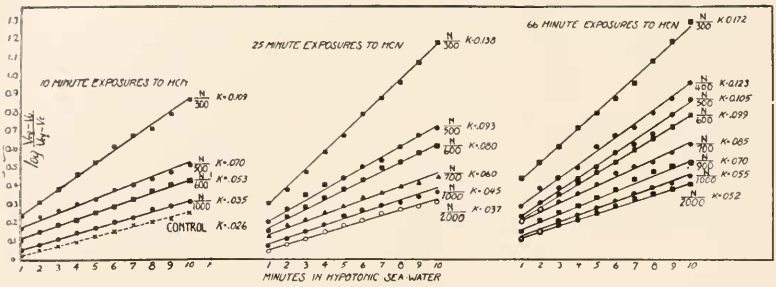

FIG. 4. Log $\frac{V_{eq} - V_0}{V_{eq} - V_t}$ (in which V_{eq} is volume at equilibrium, V_0 is volume at first instant, in sea-water, and V_t is volume at time t), plotted against the time in 50 per cent. sea-water. K, the velocity constant, is obtained from the slope of the lines.

HCN for any given length, the greater is the rate of swelling. Fig. 5 shows the rate at which the velocity constants increase with increase of concentration and increase in time of exposure to the HCN solutions. Fig. 6 is a composite curve in which all the

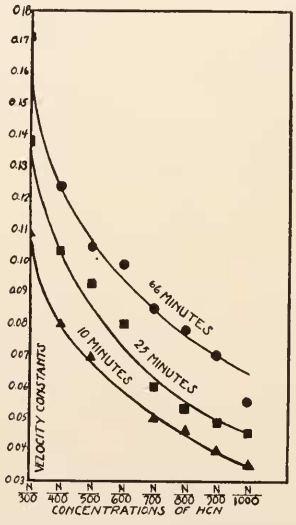

FIG. 5. Showing the rate of decrease of velocity constants with decrease in concentration of HCN.

velocity constants have been plotted to form a single curve. The various velocity constants have been plotted against their respective concentrations. The figure shows that the increase in the rates of reaction proceeds at a regular rate whatever the concentration of HCN and length of exposure.

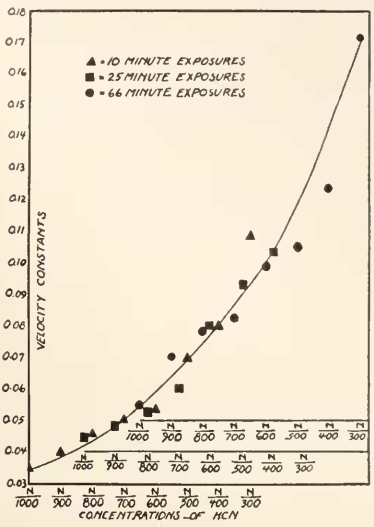

FIG. 6. All the velocity constants plotted against their respective concentrations to show that the velocity constants increase at a regular rate with increase in concentration of HCN and increase in time of exposure to these concentrations.

DISCUSSION.

Lillie's ('13) explanation of the phenomenon of antagonism by various anæsthetics and of anæsthesia in general is based on his experiments which showed that in every case the anæsthetics which he used prevented a general increase in permeability brought about by various salts which are toxic to the cell. That this decrease in permeability by anæsthetics does not always follow, at least when HCN is used as an anæsthetic, is shown by the preceding experiments. Heilbrunn ('25) has also found that ether in various concentrations increases the permeability of *Arbacia* eggs to water. The difference in the results obtained by Lillie and those reported in this paper may possibly be due to the difference in types of anæsthetics used. HCN, as is very well known, dissociates only very slightly. That it enters the cell as a molecule, as does CO_2 [Jacobs ('23)] and H_2S [Beerman ('24)], has been shown by Bodine ('24). Bodine suggests that HCN enters the cell as a molecule and ionizes within the cell to give an intracellular acidity even though the extra-cellular HCN solution is slightly alkaline.

It was shown that HCN is more potent as an anæsthetic than was KCN, since a greater concentration of KCN than HCN is needed to produce anæsthesia. One possible explanation of this follows: KCN in solution is alkaline due to the manner in which it dissociates:

$$KCN + H_2O \rightleftarrows KOH + HCN.$$
$$+ \quad \Updownarrow \quad -$$
$$K + OH$$

Thus it can be seen that a solution of KCN always contains a certain amount of HCN molecules. Therefore the anæsthetic property of KCN may be due to the HCN molecules present in it, but this HCN is partly antagonized, or its effects interfered with by the KOH formed at the same time. Why HCN should cause an increase and KCN a decrease in permeability is as yet problematic, unless it is the KOH in the KCN solution which is producing the decrease. It is hoped to gather more data on this point in the future.

That HCN acts primarily on the cell membrane and not on the interior of the cell, seems likely in view of the fact that eggs, whether treated with HCN or with sea-water, finally reach the same equilibrium point. From recent work Lucke and McCutcheon (personal communication) state that the velocity of swelling or shrinking of *Arbacia* eggs in hypotonic or hypertonic sea-water is the same, so that Heilbrunn's objection to calling an increase in the volume of the egg an increase in permeability on the ground that it may be an increase in the extensibility of the membrane, does not seem to hold. We would expect, from his statement, that the eggs would shrink faster than they would swell.

Summary and Conclusions.

(1) HCN in concentrations varying from $N/300$ to $N/2{,}000$ causes an increase in the volume of *Arbacia* eggs when placed in 50 per cent. sea-water, the rate of swelling varying directly as the concentration of HCN and the time of exposure to the HCN solutions.

(2) KCN in concentrations varying from $N/300$ to $N/900$

causes a decrease in the volume of the eggs when placed in 50 per cent. sea-water, the rate of decrease varying inversely as the concentration of KCN.

(3) Both HCN and KCN act as anæsthetics, the HCN being more powerful than the KCN.

(4) Anæsthetized eggs can be fertilized while in that condition but it appears that cell division is delayed until the anæsthetic has diffused out of the egg or until the narcotic condition has been reversed.

(5) The formula $kt = L_n \frac{V_{eq} - V_0}{V_{eq} - V_t}$ (where V_{eq} is volume at equilibrium; V_0 is volume at the first instant (in sea-water); and V_t is the volume at time t) correctly represents the rate of reaction.

(6) It is suggested that it is the HCN molecules present in a solution of KCN which causes anæsthesia by that salt, and that its lessened effectiveness is due to the antagonistic (?) action of the KOH which is present, at the same time, in an aqueous solution of KCN.

Appreciation is expressed to Dr. J. H. Bodine for suggesting the problem and for the helpful advice given the writer.

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THE LOW SEX RATIO IN NEGRO BIRTHS AND ITS PROBABLE EXPLANATION.

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It has sometimes been stated that the ratio of male to female births is lower among the Negroes than among whites. The records of births published by the U. S. Census Reports for 1880, 1890, and 1900, gave very low sex ratios for the Negroes,—namely 100.25, 102.67, and 99.80 respectively. The data on births for these years were admittedly incomplete. Births were estimated "by adding to the living childern under one year of age as shown by the population returns, the number of those who were born during the year ending May 31, but who died before the end of the year, as shown by the returns of deaths." The ridicuously low death rates estimated on the basis of the census returns for 1880 and 1890 made it evident that reports of deaths collected during the census year were very incomplete. The deficiencies in the census of 1880 were estimated by Dr. J. S. Billings as about thirty per cent., and those of 1890 were probably even greater. It is quite natural that failures to report deaths would affect most the data on the mortality of infants, especially among the Negroes. That this is the case is evinced by the very small proportion of infant deaths reported as compared with the number of living children under one year of age, whereas it is well known that the actual mortality rate among Negro infants is very high. Inasmuch as male infants suffer an exceptionally high death rate, the effect of basing estimates of births partly on data which leave out a large proportion of infant deaths is to give a sex ratio with too low a proportion of males. On account of the higher infant mortality of the Negroes, to say nothing of more numerous deficiencies in the records, the Negro sex ratio would be reduced more than that of the whites.

Aside from the rather unsatisfactory compilations published in the Census Reports we had, until a few years ago, relatively meager data on Negro births. Dr. J. D. Nichols has compiled records for the District of Columbia (1874–02) and finds a sex ratio in Negro births of 103.10. Beginning in 1915 the annual

reports on birth statistics issued by the Bureau of the Census furnish a sufficiently large amount of data on the sex ratio of Negro births to give very reliable statistical results. The number of Negro births in the U. S. Registration Area from 1915 to 1923 inclusive totals 397,977 males and 386,348 females, giving a ratio of 103.01 males to 100 females. For the same period and area there were born among the whites 5,985,181 males and 5,651,287 females, giving a sex ratio of 105.91. The sex ratio of native-born whites was somewhat higher, 106.072, while that of foreign-born whites was slightly lower, 105.55. The sex ratio of children of mixed marriages, native and foreign-born, showed an intermediate figure,—105.84. The ratios for the Indians and the Japanese were 106.06 and 106.72, respectively, figures very close to the sex ratios of the Caucasians.[1]

Does the low sex ratio for Negro births indicate a peculiarity of race? The comprehensive data assembled by Gini point to the conclusion that the sex ratio constitutes a remarkably constant peculiarity of the human species. Before concluding, therefore, that the Negro sex ratio is essentially different from that of the white race other explanations should be sought for. Nichols has made the plausible suggestion that the low sex ratio among Negroes is a consequence of ante-natal mortality. If there is a greater ante-natal mortality among the Negroes than among the whites, and if this mortality is relatively higher in the male sex, there would naturally be a lower sex ratio among the live births in the Negro race. It is a well known fact that the sex ratio of still-births and abortions is unusually high. Data on still-births have been published by the Bureau of the Census for only three years, 1918, 1922, and 1923. These data are naturally very incomplete, and different states have various ways of defining and recording still-births. Nevertheless the data are quite illuminating in relation to the problem in question. The still-births and sex ratios for different groups of the population are shown in the following table:

[1] The data on births for 1924, which have just come to hand, show much the same relations as those quoted. For total live births the ratio for Negroes is 103.98 and for all whites 105.95. The more recent data, therefore, bring the sex ratios for Negroes and whites more closely together. The sex ratios for still-births are as follows: total still-births, 137.48; total white, 137.96; native-born white, 137.81; foreign-born white, 145.26; native and foreign-born, 133.6; total colored, 135.17; Negro, 135.08.

STILL-BIRTHS IN THE U. S. REGISTRATION AREA IN RELATION TO RACE AND NATIONALITY.

		Total Still-births.	Total White.	Native White.	Foreign White.	Native and Foreign White.	Total Colored.	Negro	Indian.	Japanese.
1918	M.	28,127	24,837	15,489	6,413	2,148	3,290	3,257		
	F.	21,507	18,190	11,509	4,466	1,657	2,317	1,482		
1922	M.	39,672	33,593	21,150	8,254	3,190	6,079	5,925	53	91
	F.	29,173	24,739	15,785	5,829	2,374	4,434	4,332	30	59
1923	M.	39,566	33,511	21,232	7,986	3,302	6,055	5,925	37	76
	F.	29,235	24,770	15,840	5,831	2,436	4,465	4,360	32	64
Total	M.	107,365	92,301	57,871	22,653	8,640	15,424	15,107	90	167
	F.	79,915	67,699	43,134	16,126	6,467	11,216	10,174	64	123
Ratio		134.35	137.67	134.16	140.47	133.61	137.52	148.49	140.63	135.78

In all the groups, as may be readily seen, the sex ratio for still-births is remarkably high, and it is especially high for the Negro.

Now for the second point,—the relative proportion of still-births in Negroes and whites. Calculating the ratio of still-births to total births in the two races we find that the ratio is over twice as high among the Negroes as among the whites, namely 7.3 per cent. among the former and 3.5 per cent. among the latter. Syphilis, which is a potent cause of still-births and abortions, is very much more prevalent among the Negroes, and doubtless accounts in no small measure for the high Negro rate of ante-natal mortality.

The facts of differential race and sex mortality in uterine life enable us to explain the low sex ratio of live births among the Negroes without assuming that there is any real racial difference involved. In order to test this interpretation further I have added the still-births and live-births together in the two races and then calculated the sex ratios for total births. As would be expected, the differences between the sex ratios of the two races were reduced. For all whites combined the sex ratio became 106.72, and for the Negroes, 105.54. The inclusion even of the confessedly incomplete data on still-births wipes out most of the difference between the sex ratios of the two races. If complete data on still-births were available, the differences between the sex ratios would probably be reduced to insignificant proportions.

It is perhaps worth while to point out that the sex ratio of offspring resulting from the mating of native-born with foreign-born parents is lower that it is among the native-born. Such matings do not necessarily represent the union of distinct ethnic stocks to a much greater degree than the matings of either the native or the foreign-born, although they probably do so to a certain extent. If we may judge from my studies on the matings falling in this class among the parents of college students,[1] more than fifty per cent. of such mixed marriages would be between persons of the same extraction. On the whole, the federal statistics on births indicate that the sex ratio is little affected by the crossing of different ethnic stocks. There are several factors associated with educational, social, and economic status which probably influence the sex ratio to a greater degree.

The American Negro is to a considerable, but not precisely ascertainable, extent a product of the union of very distinct races. There are no extensive data on the sex ratio of mulatto births as compared with that of the more nearly pure blacks. But since mulattoes are relatively much more numerous in cities than in the country, one may compare the sex ratios of Negroes in urban and rural communities. I have done this for two years, 1922 and 1923, and have added the still-births and live births together. The sex ratio for the cities of the Registration Area is 103.78, and for the rural districts, 106.35. It would be unsafe to conclude, however, that race mixture lowers the sex ratio in this case. The relation is more readily explained by the higher pro-proportion of still-births among the urban Negroes.

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LIVE BIRTHS AND SEX RATIOS AT BIRTH IN THE U. S. REGISTRATION AREA, 1915–22, ACCORDING TO RACE AND NATIONALITY.

	Total Births.	Total White.	Native White.	Foreign White.	Native and Foreign White.	Total Colored	Negro.	Indian.	Japanese
M.....	6,412,118	5,985,181	3,791,433	1,499,368	619,295	426,937	397,977	7,389	18,443
F.....	6,064,740	5,651,287	3,574,396	1,420,482	585,110	413,453	386,348	6,967	17,281
Ratio.....	105.73	105.91	106.07	105.55	105.84	103.26	103.01	106.06	106.72

THE CHROMOSOMES OF THE INDIAN RUNNER DUCK.

ORILLA STOTLER WERNER.

During recent years the attention of a number of cytologists has been focused on the problem of the avian sex-mechanism. Their investigations have brought varying results and numerous hypotheses have been advanced to explain the cytological findings in such a way as to bring them into agreement with the genetic evidence which is beyond dispute.

Cytologists agree that both physiologically and morphologically the character of the chromosomes of the birds is undetermined and that up to this time our only method of forming a conception of the activities of the chromatic material is through genetic study. In view of this consensus of opinion it would be a hazardous thing for a beginner in the field to criticize the theories that have been developed around the studies of the avian chromosomes unless he has a sufficient number of properly prepared figures for study and has subjected them to a very critical analysis.

Stevens, Hance, and Schiwago agree that the male chick appears to have two large chromosomes equal in size. These they presume are the X-chromosomes. Guyer claims to have evidence to show that in the guinea hen and in the domestic chicken the sperm are of two classes. These are to be distinguished by the presence or absence of an unpaired X-element or accessory chromosome. He believes, however, that the sperm which do not possess the X-element degenerate and are, therefore, non-functional. In regard to this Wilson (1925) says: "In view of this fact it is remarkable that the diploid group seems to show in the female but one large curved chromosome (X) while in the male two such elements are present. The diploid group thus seems to show the expected relations, the female being heterozygous, the male homozygous; but, per contra, the gamete formation of the male seems to show this sex to be cytologically digametic, with one class of gametes non-functional. It is stated,

further, that the X-chromosome of the spermatocyte division is a bivalent body (representing the large pair in the spermatogonia) which passes as such to one pole. If these facts be correctly determined they offer a cytological puzzle with which it is not possible to deal without additional data."

For some time (June 1925 to June 1926) I have been engaged on a study of the chromosomes of the Indian runner duck and I offer the following data as a partial solution of the avian sex-chromosome problem.

Material and Methods.

The material used for giving mitotic figures consisted chiefly of the embryonic membranes of both sexes and to some extent, the germ cells of the male. In most cases the cells of the amnion afforded the best examples, although the cells of the chorion and the allantois gave good results; but such material is more difficult to prepare.

Tissues were obtained from individuals at different stages of incubation ranging from five to twenty-one days. However, those from eight to eleven days were found most suitable for the purpose. Before the eighth day stage it is difficult to determine sex and after the eleventh day the amnion has so completely formed that few dividing cells can be found.

Four general methods of technique were employed; whole mounts from embryonic membranes, stained sections of the embryos, sections of the testes, and smears of the testes.

Membrane Technique.—Of the embryonic membranes the amnion was found to give the best results. This is a very delicate tissue resembling a silk chiffon veil. Because of its delicate structure it was found best to keep it as nearly intact as possible. In this way the pressure of the amniotic fluid kept it stretched while being fixed and thus most of the tissue could be saved. Of the allantois only parts could be used. This was especially true of embryos of longer incubation, for the heavy blood vessels catch and hold the stain and the tissue is thick and becomes hard.

Great care was taken that the eggs should not become chilled while being removed from the incubator. The embryo within its amnion, and sometimes a part of the allantois, was removed from

the egg with warm instruments and placed in Allen's modification of Bouin's fluid for two hours. The temperature of the fixative was kept at 37°. The amnion was punctured at the end of an hour to allow the fixative free access to the embryo.

The tissues of the first three embryos were rendered practically useless by increasing the strength of the alcohol too rapidly. The chromosomes were clumped and massed so that it was almost impossible to make a count. For this reason the following procedure was worked out. When the tissues were removed from the fixative they were rinsed in several changes of distilled water at a temperature of 37°, then passed successively through the following grades of alcohol: ½, 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 per cent. During this time the alcohols were maintained at the same temperature and the tissues were allowed to remain approximately twenty minutes in each fluid. While the material was in the 10 per cent. alcohol the membranes were removed from the embryos, and placed in a 2 per cent. solution of iron alum; one and one half hours. They were then rinsed in several changes of distilled water and placed in an aqueous solution of Heidenhain's hematoxylin, two hours; rinsed in tap water and destained in iron alum. They were then passed successively through the following grades of alcohol, remaining about ten minutes in each grade: 12, 14, 16, 18, 20, 22, 25, 30, 35, 40, 45, 50, 55, 60, 70, 80, 90 and 100 per cent. They were then passed through xylol, fifteen minutes, cedar oil three hours, back to xylol ten minutes, 100 per cent. alcohol one hour, forward to cedar oil two hours, then xylol fifteen minutes, and finally cut into small pieces for mounting. The double clearing makes the tissues beautifully transparent.

As suggested by Painter in his study of mammalian material, the large mesodermal cells were found to be the best for study. For this reason the tissue was placed on the slide with the mesodermal surface upward. They were mounted in gum damar, a small leaden weight being placed on the cover slip while the slides were drying.

Technique for Embryos.—The embryos were taken from the ten per cent. alcohol and passed through the same grades as were the membranes but because of their greater bulk were left thirty minutes in each grade. They were then passed through half

100 per cent. alcohol plus half xylol, 15 minutes; pure xylol, five minutes; one half xylol and one half 48° paraffin, fifteen minutes; then embedded in the usual way. They were cut at seven micra and stained with Heidenhain's hematoxylin and the work completed as is usual for such material. These slides were used in determining the sex of embryos whose membranes had furnished cells for study.

Technique for the Sectioned Material of the Testes.—The testes were from eight-months-old individuals. On removal from the body they were cut into millimeter cubes and dropped into the same fixative as above as soon as possible. They remained in the fixative two hours at 37° then were washed in several changes of distilled water at the same temperature. The material was now passed through the following grades of alcohol, two minutes each: ½, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 15, 18, 20, 22, 25, 30, 35 and 40 per cent. At this stage all the alcohol was drained off except enough to cover the tissues. Then by means of a pipette, equal parts of bergamot oil and 50 per cent. alcohol were dropped in. The mixture was stirred constantly by bubbling it with a pipette. Again all the liquid was drained off except just enough to cover the tissues, and in each of the following changes this rule was observed. Equal parts of 60 per cent. alcohol and bergamot oil; equal parts of 70 per cent. alcohol and bergamot oil; pure bergamot oil. The old oil was now drained off and fresh bergamot oil dropped in. This process was repeated several times until all the alcohol was removed. The tissues were then left in the pure bergamot oil two hours (the tissues may be left in this oil for several hours without injury). The oil was then drained off and three fourths parts of bergamot oil and one fourth part of oil of wintergreen were dropped in, then one half bergamot and one half wintergreen, one fourth bergamot and three fourths wintergreen, pure wintergreen. Then the old wintergreen was drained off and fresh added several times until no bergamot remained.

From the oil of wintergreen the tissues were carried by steps through to paraffin. Sixteen bottles were used, starting with one part paraffin and fifteen parts wintergreen and finally ending in pure paraffin. Then the tissues were passed through several

changes of pure paraffin to remove all the wintergreen. They were then placed in an oven for twelve hours, then in fresh pure paraffin for one half hour and finally embedded. While the tissues were being passed through the paraffin, the temperature was kept just so the paraffins would stay liquid, care being taken not to raise it above this point. Sections were then cut about five and one half micra. I think, however, that it would be better to cut them thicker than this, say about seven or eight micra. In this way many more cells could be found with none of the chromosomes sectioned away.

After the tissues were mounted the work was completed according to the usual methods.

The chromosomes in these cells stand out clear and beautiful.

The destaining is a delicate process and must be done with extreme care. No definite time can be given for the iron alum in destaining for the time depends upon the thickness of the sections, the concentration of stain used, etc. Experience alone brings the desired results. The work is best done under the low power of the microscope (10× ocular and 2/3 objective).

Technique for Testis Smears.—These were prepared just as the sections but the oils and the paraffins were omitted. From the 40 per cent. alcohol they were passed successively down through the grades of alcohol, care being taken to gradually lower the temperature of the fluids until when the work is completed they are at room temperature. This can best be accomplished by placing all the containers on an electric embedding plate.

The study of the avian chromosomes has presented difficulties in that the chromosomes are easily massed together, making a count difficult or impossible. With the technique here employed this difficulty has to a considerable degree been avoided.

Approximately six hundred cells were examined. The greater portion of these were soma cells from the tissues of nine individuals. In determining the number of chromosomes I have, in most cases, made drawings from several different slides. However, where the cells were found particularly clear and distinct I have made several drawings from the same slide.

The chromosomes were measured in the following manner. A separate camera lucida drawing was made of each. A thread

was moistened and laid along the median curvature of the chromosome outline. By means of a razor blade its ends were cut at points corresponding to the boundaries of the ends of the chromosome as the thread lay upon the drawing. The segment was then removed and drawn out straight and measure taken in millimeters. In this way with a considerable degree of accuracy the length of the chromosomes was obtained.

Chromosomes of the Somatic Cells.

In the duck it will be more convenient to consider first the complex of the male (Figs. 1 to 7 and 43 to 48) since it is in this sex that the number of chromosomes is even.

There are probably seventy six. Some deviation from this number was found in the early part of the work. This was due, in greater part, to a failure to recognize the small globe-shaped chromosomes. For a time they were thought to be basophylic knots on the filaments that sometimes occur between the smaller chromosomes. As the work progressed, however, it became more apparent that these were true chromosomes. In addition to this deviation about half a dozen cells were found in the amnion in metaphase that appeared to have but half the usual number of chromosomes (thirty-eight). At this time no explanation can be offered for this condition. With the exception of the above few cases all cells examined, in which the chromosomes were well separated, seemed to have seventy-six. I am inclined to think that this is the basic number.

These seventy-six chromosomes fall readily into three general groups: six pairs of large chromosomes, including three J-shaped and three rod-shaped; nine pairs of short rod-shaped chromosomes; and twenty-three pairs of globe-shape. This may be seen in Figs. 43–48, Plate 8, which is an artificial pairing of these chromosomes arranged according to size. The morphology and the length were taken as a basis for this pairing. Where there is a slight difference in the lengths of the members of a pair, the shorter member is usually broader at one end than its mate. This makes the chromatic material of each member approximately the same.

Of the six pairs of large chromosomes (Nos. 38–33) the largest

are J-shaped bodies (Figs. 3, 4, 6, 7 number 38). Next in size are a pair that are usually of rod-shape (Nos. 37) only slightly shorter than the longest in the complex. Pairs 36, 35, 34, and 33 form a graduated series in which there is but little difference in the lengths of the consecutive pairs (Figs. 1–7 and 43–48). However, there is a greater difference between pairs 34 and 33 than between the other pairs. Pairs 36 and 35 are of the J-type (Figs. 5, 6, and 7), and 33 and 34 are usually of rod-shape or bent rods, though in some prophase cells they seem to be J's (Figs. 3, 7, 44 and 45).

The J-chromosomes (Nos. 38, 36, 35) of this group are, in most cases, constant in shape throughout prophase and metaphase and, as far as observed, in anaphase. The three pairs of rod-shaped chromosomes (37, 34, 33) assume various forms according to the stage of mitosis and according to their position in the nucleus. The members of the largest pair (Nos. 37) are especially interesting in this respect. In the metaphase they are, in most cases, straight rods pointed at the proximal end (Fig. 3). At times, however, they appear in the form of U's (Fig. 4). If they lie near the nuclear wall they bend to accommodate themselves to this limitation. One or both ends may be bent (Fig. 7). Also these two bodies are not always bent in the same shape in the same cell but are so nearly the same in size that one may recognize them among the other chromosomes with a fair degree of certainty. Fig. 23 shows some of the shapes that they assume. There seems to be some differentiation of the chromatic material near the ends of these chromosomes which gives them increased flexibility at these points. This differentiation seems to be *a* suture accompanied by a slight constriction of the chromosomal wall. One of these sutures, if it is such, is near the incurved end when the chromosome lies as represented at *a*, *b*, *c*, *d*, in Fig. 23. The other point of differentiation is at the opposite end of the chromosome and marks off a portion of the chromosome about three times the length of the former end part.

With reference to the matter of sutures and constrictions, the condition found is not peculiar to the duck for such has been described by many observers in both animals and plants. These sutures and constrictions, it seems, may be median or at any

other point and in some cases at least, they are constant in position. For example, Sakamura ('16) found that in *Vicia* several of the chromosomes show a constant subterminal constriction and that those of one pair show a constant median constriction in addition. However, Agar ('12) found in *Lepidosiren* that these sutures vary in position in different chromosomes and that they correspond with the points of attachment to the spindle and that their position in the chromosome of the meiotic division corresponds with that in the spermatogonial chromosomes. Wilson, however, points to the fact that these sutures and constrictions are visible in the prophase before the spindle is formed and therefore are not caused by the attachment. That these sutures may not be necessarily connected with the attachment of the traction fibers is shown by the No. 37 chromosomes of my material. Here there are two sutures, one at about one twelfth of the length of the chromosome distant from one end and the other at about one fourth the length of the chromosome distant from the other end. It is hardly probable that there would be two traction fibers so widely separated arising from the same somatic chromosome.

Again it should be pointed out that the permanence of position of such sutures at at least two different points along such chromosomes (see Fig. 23) would seem to be morphological evidence that such chromosomes differ in a qualitative way throughout their length.

So far, we have been following the characteristics, sizes, etc., of the largest chromosomes. Let us next consider the characteristics of the chromosomes of the intermediate group. There are nine of these (Nos. 32 to 24) and they are of the short rod-shape type. Among these is one pair, the members of which are sometimes seen as crescent-shaped bodies (Fig. 8, cr.). Altogether the nine pairs form a closely graduated series ranging in size from those somewhat shorter than the shortest in the foregoing series to short ones that are almost as thick as they are long, but certainly distinguishable as rods by their acute angles (Figs. 43–48).

The third group of chromosomes consists of forty-six round or globe-shaped bodies (numbers 23–1). These also form a closely

graduated series. Only twenty-two of these are of sufficient size to permit their being paired. The remaining twenty-four are small and so closely graduated in size that it is impossible to pair them with any degree of certainty.

The nine pairs of short rods and the twenty-three pairs of globe-shaped chromosomes are remarkably constant in shape during the progress of prophase from the time they are discernible as individual bodies until they enter the equatorial plate in metaphase. Here they mass together to such an extent that frequently they are no longer distinguishable as individual bodies. But even in this phase it frequently happens that they may be seen in their characteristic forms.

There appear to be seventy-seven chromosomes in the cells of the female (Figs. 8–22, 37–42). The longest of these is a large unpaired body, larger than any of the other chromosomes, and on account of its size, in most cases, easily distinguishable from the other chromosomes (Fig. 37–42, W). In cross section it is large at one end and taper at the other. In the earlier stages of mitosis it is a more or less bent rod, seeming to accommodate itself to the other nearby chromosomal regions and also to the nuclear wall (Figs. 8, 10, W). In the late prophase and the early metaphase it continues to be a more or less bent rod (Figs. 9, 11). In Fig. 17 it is a rod bent upon itself. Figs. 14 and 22 show it as **U**-shape while 16, 19 and 20 show it with bendings in different regions. In Fig. 12 it is a rod somewhat foreshortened. It would seem that this chromosome possesses a great degree of flexibility throughout its length which permits its bending in various ways.

The next largest chromosomes in the cells of the female take at times the form of curved rods but more frequently are of **J**-shape (Figs. 8–22, 37–42; 38w, 38Z). Whether they are gonial mates or not it is difficult to say. Theoretically, as will appear later, they should not be. From their size and their **J**-shape one would suppose that at least one of them is a homologue of the 38's in the cells of the male. The other might be of the nature of a w-chromosome.

Slightly shorter than these are two large curved rods (Nos. 37) one of which in prophase and early metaphase quite frequently takes the form of an **S** (Figs. 9, 17). The members of this pair

resemble the pair number 37 in the cells of the male in sutures and constrictions (Fig. 23 a–d), and like them they assume various shapes (Fig. 23, e–i).

The remaining seventy-two chromosomes in the cells of the female (Nos. 36–1, Figs. 37–42) are approximately the same with respect to behavior, size relations, etc., as those in the cells of the male (Figs. 42–48).

In my study of the somatic cells I have been increasingly impressed with the evident pliancy of the chromosomes. They appear to bend to accommodate themselves to the nuclear wall and to other chromosomal regions. In most of the chromosomes (with certain exceptions to be mentioned later) the degree of pliancy seems to be about equal in all their parts. For this reason the morphology of chromosomes of the same length which are probably synaptic mates is not always the same. The ultimate conclusion regarding the shape of each particular chromosome, I believe, must be drawn from its appearance in metaphase where it is entering the equatorial plate and even in this phase some may be atypical in shape because of the obstructions of other chromosomes. The atypical forms, of course, have been found a lesser number of times and the conclusions are based on the larger number of cases.

The most convincing evidence is found in a comparative study of the chromosomes when those from different cells are arranged in serial order and in tables as in Plates 7, 8, and 9. Figs. 37 to 42 show the chromosomes from cells of the amnion of females; Figs. 43 to 48 from the cells of the amnion of males; Figs. 49 to 54, Plate 9, show first-spermatocyte chromosomes from smear preparations of adult testes. In these tables the constancy of the size and shape of the individual chromosomes stand out at once. There is some variation in form of the chromosomes from cell to cell but it is due, I believe, as previously stated, to their pliancy and in some instances to the particular angle at which the chromosome lies relative to the observer, and probably more than anything else to the particular stage of mitosis in which the cell happens to be found. Figs. 37, 38 and 40 are taken from early prophases (Figs. 8, 9, 10); Figs. 39, 41 and 42 are from prophases of later stage (Figs. 13, 12, 16); Fig. 43 is from a cell in early

metaphase (Fig. 4); Fig. 47, and 48 are from late prophases (Figs. 1, 2).

The morphological groups and the size relations within the groups are very evident in this comparative series. The distinction in size, etc., between the group of twelve (thirteen in the female) large chromosomes (38–33) and the group of 18 of intermediate size (32–24) is clearly marked in Plates 7 and 8. This is especially true in Figs. 37, 38, 40, 43, 44 and 45. It may also be plainly seen that the intermediate-sized and the globe-shaped chromosomes each form a finely graduated series. Because of this finely graduated condition and because there are so many of them there may have been some inaccuracy in the pairing and, in the case of the globe-shaped series, possibly in the count also, for these, the smallest chromosomes, frequently overlie one another. But it is certain that there is no inaccuracy in the count in the cases of the members of the larger and of the intermediate-sized groups. There is clearly 12 plus 18, or 30, of these bodies in the cells of the male and 13 plus 18, or 31, in the cells of the female. Neither can there be any doubt as to the presence of at least one unpaired chromosome among those from the female, for it is larger than and morphologically different from the other chromosomes. In the case of the chromosomes which have been arbitrarily numbered 38w and 38Z it might be said one is dealing with gonial mates, since they are similar in length and contain approximately the same amount of chromatin. These facts, however, considered alone, are not sufficient proof that they should be considered homologous rather than that they might be, as I shall attempt to prove later, two additional odd chromosomes which are concerned with the sex-mechanism. The important point in this paper is the fact, previously indicated, that a study of the cells of the male reveals an even number of the larger chromosomes while a study of the cells of the female shows an odd number and one more than in the cells of the male.

The morphological difference of the last group of chromosomes (globe-shaped) from the two preceding groups is not a condition peculiar to the duck. Painter shows the same condition in the lizard (Figs. 35, 36). As previously stated the twenty-two smallest chromosomes are so small and so closely graduated as to

size that it is impossible to pair them. I have placed them in these plates simply to finish the series. In the first spermatocyte cells (Figs. 24–29) these globe-shaped chromosomes are picked out with less difficulty because they are fewer in number; but here the tetrad condition often interferes with distinguishing size relations. However, where a large number of such cells are studied a considerable degree of certainty concerning numbers, morphology, and size relations may be obtained.

Chromosomes of the Spermatocytes.

The preliminary process of reduction is initiated by syndesis in the course of which the chromosomes become closely associated two by two to form bivalents or gemini. This is, of course, only a pseudo-reduction producing a haploid group of bivalents each representing a pair of chromosomes. Because of this fact we would expect to find in the first spermatocytes where such phenomena occur one half the somatic number of chromosomes.

In counting, the prophase stages were found to be most suitable. Here the chromosomes lie well apart, which facilitates, not only the counting, but also a study of the forms of each, especially of the large tetrads (Figs. 24–29). Smear preparations were most suitable because whole cells could be used with the resulting certainty that all the chromosomes were present. Metaphase figures were also studied but no drawings of these are here used on account of the tendency of the small tetrads to crowd together in the center of the plate. The large chromosomes, however, stand out clearly in metaphase and have a tendency to occupy the outside of the circle as in the somatic cells (Figs. 1–22) and as in similar cells of other species (Figs. 35, 36).

Figure 24 represents a particularly clear prophase. In Fig. 49 the chromosomes of this cell are artificially arranged according to their size. In these bodies the tetrad form can be made out in most of the larger members, and at least the bivalent condition may be seen in the smaller members of the series. This is also true of Figs. 50 to 54. Attention is called to the similarity of the size relations among the members of the spermatocyte series to that of the homologous pairs in the somatic series (Plates 7, 8).

There is a distinct drop in size between the sixth and the seventh largest tetrads (Nos. 33 and 32). The same size distinction may be noted between numbers 33 and 32 of the soma cells. There is a similar distinction between the 35's and the 34's in the spermatocyte and somatic series. The size relations among the shorter spermatocyte rods (Nos. 32–24) and the bivalents derived from the globe-shaped chromosomes are apparently about the same as in the soma cells. In each case they form a similarly graduated series.

In addition to the resemblance in size between the spermatocytic and the somatic series there is also a striking similarity in form. Tetrad number 38, the largest in the group (Fig. 24), shows that it has arisen from a pair of **J**'s similar to those seen at 38Z in Fig. 43, Plate 8. In Fig. 25 the long arms of the **J** have not yet disjoined; the shorter arms have. This chromosome is well shown in Fig. 52 where the disjoining is almost complete. Chromosomes 36 and 35 also show that they have arisen from a pair of **J**'s (Fig. 25). In each of these chromosomes the long arms of the **J**'s have not disjoined while the shorter ones have. Chromosome number 37 (Figs. 24, 25, 26, 27, also Plate 9) shows constrictions similar to those found in number 37 in the soma cells, although these are more apparent in the tetrads, *i.e.*, the constrictions are deeper. In some of these cases only one of the constrictions is apparent but whether one or both are present, when they do appear, each seems to be in about the same position in the members making up the tetrad that it would occupy in the somatic homologues.

Gonomeric Grouping.

The probable existence of an odd chromosome in one of the gametes, as well as a definite number of the large chromosomes in each of the gametes, is further evinced in somatic cells in the grouping of the chromosomes in early metaphase. I have examined numerous cells in metaphase in both sexes. In the cells of the male the large chromosomes are grouped six on one side of the forming equatorial plate and six on the other (Figs. 3, 5, 6). In the cells of the female there are six on one side of the plate and seven on the other (Figs. 17, 18, 19, 20, 21, 22). In every case

there is in the group of seven, one chromosome which is larger than the others which has the characteristic form of the largest odd chromosome in the cells of the female, large at one end and taper at the other.

Schiwago (1924) has recognized gonomeric grouping in the cells of the domestic chicken. (I have taken the liberty of reproducing some of his drawings as well as those of Hance from the same form and two from Painter from the lizard). The lettering of the chromosomes in Figs. 32 and 33 are as Schiwago gave it. The same grouping is apparent in the work of Hance and Painter although the authors do not point out the fact. I have again taken the liberty to draw a line through each of these figures (Figs. 30, 31, 35, 36) which might suggest a possible separation of the chromosomes into maternal and paternal groups.

D. H. Tennent (1908) found gonomeric grouping when working on the eggs of *Toxopneustes* and *Arbacia* fertilized with *Moina* sperm. Margart Morris (1914) in her work on hybrids between *Fundulus* and *Ctenolabrus* found a somewhat similar condition. Many other instances of this phenomenon among different species are on record.

Gonomery represents no more than a tendency on the part of the chromosomes to remain in separate maternally and paternally derived groups during a part of the early development. Considering these facts, such a phenomenon in the duck would seem to afford a valuable check on the count of the chromosomes, as well as a check on the number contributed by each sex, especially with respect to the large chromosomes, since these are so well defined that they may be readily recognized.

Filamentous Linkage.

Filaments which seemed basophylic in character were found connecting chromosomes in somatic cells in prophase stages. Not all groups of chromosomes formed by such fastenings were constant. Some groups composed of definite numbers were found a sufficient number of times to justify one in concluding that they might be relatively constant. In nearly all cases the groups are made up of chromosomes of the intermediate and smaller

sizes. In some cases chromosomes especially of the short rod-type might be connected by two such filaments in such a way as to extend one from either corner of the end of an individual chromosome and attaching in like manner to the end of the next chromosome. In other cases both filaments might be fastened at the same point on a rod-shaped or a globe-shaped chromosome (Fig. 10).

Of the inconstant groups one composed of eighteen small chromosomes may be seen in Fig. 9. In the upper part of this figure there is a group of two chromosomes attached to one of the large J-shaped chromosomes. Fig. 13 shows a group of 13. Sometimes filamentous fastenings occur between the smaller chromosomes when these bodies lie in what is, apparently, a linear arrangement. However no cells have been observed in which all the chromosomes so arranged were fastened by filaments. If such a condition exists, it would be exceedingly difficult to discover since the chromosomes so arranged lie close together and are usually at two foci, the one lying over the other or nearly so.

Of the groups having the more constant types of fastenings one may be seen in Figs. 1, 9, 10 and 12. In this group ten of the chromosomes belonging to the intermediate series, seem to be connected end to end by interchromosomal filaments in such a way as to form two chains of five chromosomes each, consisting each of two rods and three globes. The members of the one series are similar in size to those of the other group. There are also in late prophase, four chromosomes belonging to the intermediate series connected end to end in such a way as to form two chains of two chromosomes each (Figs. 1, 9, 12).

Quite often the greater number of the small chromosomes appear to be in linear arrangement, I believe that in some cases they extend in a circle within the nucleus. In late prophase there seems to be a row extending almost across the space within the circle of the large chromosomes (Figs. 8, 9, 12). In some cases this row bends a little as a line appears when it extends around a globe. Manipulation of the fine adjustment shows that another row similar to this one lies at a deeper focus (Fig. 12) apparently at the opposite side of the nucleus. (It must be borne in mind

that I was examining entire cells, not sections of cells.) These rows may extend in any direction across the nucleus, but they always lie within the circle of the larger chromosomes.

Discussion.

The chromosome number in the somatic cells and early germ cells is traceable half of it to the paternal parent and half of it to the maternal parent. There is, then, in the typical cell, an even number of chromosomes. If in any form an odd number is found, the question is immediately presented as to the disposition of the odd chromosome in maturation and what part it might play in the transmission of hereditary factors.

An attempt to solve the problem of the odd chromosome in the case of the Indian runner duck, as well as in any other form, necessitates not only a tracing of its disposition or probable disposition in maturation and zygote formation but also a discovery of a parallelism with the vast amount of results obtained from experimental breeding.

In the Indian runner duck the male has an even number, seventy-six chromosomes, but the female has an odd number, seventy-seven, and strange to say, one more rather than one less than is present in the male. Of course where the odd number occurs in the female, one would immediately look to the chromosomes in polar body divisions for an explanation. The difficulty of getting eggs in the right stage of maturation is at once apparent. It seems best, therefore, at this time to attempt an explanation of the phenomenon from indirect evidence and in the light of the results of the work on Lepidoptera, in which the female is known to be digametic for sex as is probably the case in the aves.

In the duck the additional unpaired chromosome present in the cells of the female is without a homologue in the cells of the male and of course could not then be a sex-linkage chromosome. If it is not concerned with sex-linkage it must probably belong to the class of sex-determining bodies known as the W-chromosomes, occurring only in females.

With this chromosome (W) ruled out of consideration it then becomes the problem to find which one of the remaining chromo-

somes in the female is concerned with sex-linkage and finally to account for the presence of a third non-homologous body which must evidently be present.

Sex-chromosomes carrying sex-linked characters are usually among the largest of the complex. In the male of the Indian runner duck, then, it is reasonable to suppose that the sex pair would be among the largest if not the largest in the series. In this series there are certainly six pairs that are sharply marked off in size from the remaining thirty-one pairs. Among them again three pairs may be distinguished as larger than the remaining three, and the largest of these is a pair of J's which it might be supposed are the sex pair. For the sake of argument, at least, let us assume that 38Z is the sex pair (Figs. 37–42, number 38Z).

Now, in the female the sex-chromosome concerned with sex-linkage should be an unpaired body and similar in size and shape to the sex pair in the male with the members of which it, of course, would have to alternate. Evidently the largest chromosome (W) can not be this body. If the sex-linked chromosomes in the male are the largest in the series, as is usual in animals, in the cells of the female we would of necessity look for a homolgue of them among the largest in the series after the longest chromosome (W) is ruled out. From the drawings it would seem that there are four of these next largest chromosomes and they are very much alike in size. Just what one of the four might function in the capacity of a sex-linkage body is not possible to determine now. It might be supposed, however, that, as in the male, it is one of the largest (38Z).

If that be the case there would then remain an unpaired chromosome in the female to be taken from the remaining three large chromosomes. This chromosome like the largest one (W) in the female series would have no homologue in the male series and of course would be limited to the female line and would be a second w-chromosome (w).

In the female complex, then, there would be a group of three large chromosomes, one of which would be a sex-linked chromosome, homologous to and capable of alternating with the largest sex-linked chromosomes in the male. The other two would be chromosomes not concerned with sex-linkage and, since they

remain in the female determining gametes, would be concerned only with sex-determination.

If the large unpaired chromosome in the cells of the female is one of a complex of three that go to make up the sex-determining mechanism, the condition in the duck might then be a case similar to that in *Phragmatobia*, one of the Lepidoptera, which was described by Seiler (1914).

In *Phragmatobia* during polar body formation a single element was seen to separate from a large heteromorphic element that consisted of two unequal chromosomes yoked together. After separation the heteromorphic element fragmented into two unequal chromosomes at one pole. As the result, half of the eggs got the large single element and half got the two unequal chromosomes.

If the duck be like *Phragmatobia*, the unpaired chromosome here designated as Z might be thought comparable to the large element in *Phragmatobia* and some two of the other large chromosomes, here designated W, w, might be comparable to the heteromorphic element in *Phragmatobia* which was seen to be distributed to one pole in maturation and to break into two chromosomes.

Sex-Determination and Sex-Linkage.

Wilson (1925) is of the opinion that "all the difficulties" in regard to an explanation of the function of sex-chromosomes in sex-determination" disappear is we assume that in any particular species there is but one kind of X-chromosome, in itself neither male-determining nor female-determining, but so adjusted to the general mechanism of development that when single it swings development toward the male side, when double toward the female side. This view essentially quantitative ascribes to the egg the capacity to produce either the female or the male, according to the presence of more or less of the X substance."

On the other hand, Bridges (1922) from his study of triploids in *Drosophila* concludes that "both sexes are due to the simultaneous action of two opposed sets of genes, one set tending to produce the characters we call female and the other to produce the characters called male." According to his hypothesis there is a preponderance of male tendency genes located in the autosomes

and a preponderance of female tendency genes located in the X-chromosomes. In the presence of one X the male-tendency genes located in the autosomes overbalance the female tendency genes in the single X-chromosome and a male results; on the contrary the presence of two X-chromosomes overbalances the male-tendency genes in the autosomes and the development is thrown toward femaleness.

Goldschmidt (1923) in his work on moth hybrids has come to the conclusion that the female producing factors lie in the W-chromosome. In crosses between certain races females are gotten through non-disjunction in such a way that the W-chromosome comes from the father and the Z-chromosome comes from the mother, just the reverse of the usual method. In such cases he finds that femaleness follows the W-chromosome.

In the light of the results obtained by Bridges on sex-intergrades in Drosophila produced through triploidy or otherwise unbalanced conditions of chromosomes, it is very clear that sex is not controlled entirely by a particular chromosome either singly or doubly represented as Wilson would say. There may be genes in many of the autosomes as well as in the sex-chromosomes which individually tend to throw the balance toward maleness or toward femaleness. But Bridges was dealing with an X–Y type of sex-determination and with male digamety. His opposing tendencies are located in the X-chromosomes and the autosomes. In addition to this his X-chromosomes carry sex-linked characters. Bridge's scheme as may be seen, will not work for the birds or moths where female digamety occurs But if we adopt the suggestion of Goldschmidt (1923) that the W-chromosome which passes from mother to daughter carries. female-tendency genes only and in addition assume that male-tendency genes are located in the Z-chromosomes of which two occur in the male and one in the female, we have a mechanism that in many cases would seem to take care of both sex-linked characters and sex-determination.

In the duck then, if the large unpaired element in the cells of the female is one of a complex of three that go to make up the sex-determining mechanism, the following scheme would be entirely in accord with the parallelism of cytology and experimental breeding.

In the male there are seventy-six chromosomes. It would seem then that seventy-four of these are autosomes and two are Z-chromosomes. In maturation each sperm would get thirty-seven autosomes, plus one Z-chromosome, or thirty-eight in all, and all male gametes would therefore be alike.

In the female there are seventy-seven chromosomes. Comparing these with the conditions in the male it would seem that there are seventy-four autosomes, plus one Z, like those in the male, plus two W-chromosomes which are not present in the male.

In maturation it might be supposed that the two W's become linked together and that one of these might pair with the Z-chromosome, the whole resulting in a tripartite body. If the thirty-seven pairs of autosomes behave as in the male there would result gametes in the female of two sorts (Figs. 55, 56) one having thirty-seven autosomes plus the Z and the other having thirty-seven autosomes plus the Ww pair. At the formation of the zygotes then, those eggs having thirty-seven autosomes plus the Z, fertilized by a sperm having thirty-seven autosomes plus a Z would result in a zygote having seventy-four autosomes plus two Z's or a male. Those eggs having thirty-seven autosomes and two W's fertilized by a sperm as before having thirty-seven autosomes plus a Z would result in a zygote having seventy-four autosomes plus one Z and two W's or seventy-seven chromosomes and would be a female.

In sex-linkage the Z-chromosomes alone would be concerned. If the double lined Z, represented in Fig. 55 as occurring in the male, is thought of as carrying a dominant sex-linked character and the single lined Z here represented as occurring in the female, be thought of as carrying a recessive sex-linked character, then in the F_1 generation, both males and females would show the dominant sex-linked characters and would be like the male of the parental generation. But in the F_1 generation all males would be heterozygous for the sex-linked characters and would form gametes of two sorts, half carrying the dominant sex-linked character and half the recessive.

In the F_2 generation all males would show the dominant sex-linked character, but of the females of this generation, half

would show the dominant sex-linked character and half would show the recessive character, the female inheriting it in each case from the male parent of the F_1 generation.

According to this scheme it might be supposed that in the case of the Indian runner duck each autosome sustains a balanced condition of the male and female-tendency genes. The Z-chromosomes might be thought of as carrying a preponderance of male-tendency genes. An individual, then, receiving two Z's would become a male. The two W's might be thought of as carrying female tendency genes only. These, however, would be thought of as being quantitatively greater than the male tendency genes carried in a single Z and an individual receiving a Ww plus one Z would become a female.

A large number of animals belong to the X–Y type in sex-determination. In these the female has an even number of chromosomes, the X-chromosome being paired or diploid. The male has one less than the even number of chromosomes found in the female, the X being unpaired. This gives the female more chromatin than the male. In a few of the animals of the WZ mechanism, such as some of the moths, the male has an even number of chromosomes. The female has the odd number and one less than the male which would give the male more chromatin than the female. But in most of the cases in the moths the female has one more chromosome than the male, the male having the even number.

In the birds, if my observations are borne out on other species, it would seem that the female has the preponderance of chromatin. If the conditions in the duck are representative of that which one might expect to find generally in the aves, we would expect in the moths which are likewise digametic in the female, that the true state of affairs would be like those moths in which the female has one more chromosome than the male. The difference between the two types of animals, the XX–XY and the WZ–ZZ, is essentially in the matter of which sex produces the two sorts of gametes. And so far as this mechanism is concerned it is a matter of indifference which sex produces the two sorts of gametes. The important thing seems to be that one sex, usually the female, should have more chromatin than the other.

Summary.

1. This study was undertaken with two points in view: First, to determine the number of chromosomes and second, to discover the mechanism for sex-determination and sex-linkage in the duck as a representative of the aves.

2. Material from thirteen individuals was used, seven males and six females.

3. Chromosome counts were made on entire cells, both somatic and spermatocytic.

4. In the somatic cells there appear to be seventy-six chromosomes for the male and seventy-seven chromosomes for the female. There is present in the cells of the female a long unpaired chromosome which is not found in the cells of the male. There is reason to suppose that there are probably among the remaining six largest chromosomes two more unpaired chromosomes, one of which, the largest, is probably homologous to the largest pair (sex-linkage) of chromosomes in the male complex, while the other, it is thought may be some one of the five remaining long chromosomes.

5. There appear to be 38 bivalents in the primary spermatocytes of the male. These agree with the pairs of somatic chromosomes in size gradations. Most of the chromosomes in the spermatocytes appear to be bivalent or tetrad in form.

6. Sutures accompanied by constrictions seem constant in position in at least two points in the largest rod-shaped chromosomes. These sutures seem to lend additional pliancy to these regions in the chromosomes.

7. Gonomeric grouping of chromosomes occurs in the amnion cells of the duck. It is also thought to be present in the chromosome plates of the gonial cells in the embryo of the chick and the lizard.

8. Filamentous linkage between certain of the smaller chromosomes appears to be present in certain stages of the prophase.

9. There is reason to believe that the sex-mechanism is of the WwZ–ZZ type similar to that found in the moth Phragmatobia.

10. This would give the female more chromatin than the male and yet preserve female digamety, which would bring this type into harmony with the usual conditions found in the XX–Xy type.

Acknowledgment is hereby made to Dr. W. R. B. Robertson, at whose suggestion this problem was undertaken, for advice and assistance during the progress of the work and for a sympathetic understanding of the difficulties of the task undertaken.

University of Missouri,
Columbia,
September 1, 1926.

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EXPLANATION OF PLATES.

All figures from the Duck are reproduced at the same scale. The drawings were outlined with an Abbe camera lucida at a magnification of 3,500 diameters, obtained with a Spencer 1/12 homogeneous immersion objective and a Zeiss 18x compensating ocular with draw tube set at 150 mm. and drawing made at the level of the base of the microscope. The drawings were then enlarged by means of a copying camera lucida to 7,350 diameters. Having been reduced one half in the reproduction, they now appear at a magnification of 3,675 diameters.

W, the large sex-chromosome carrying female-tendency genes only.
w, the smaller sex-chromosome which carries likewise female-tendency genes only.
Z, sex-chromosome carrying a preponderance of male-tendency genes and also sex-linked genes.
cr, cresent-shaped autosomes No. 32.
38Z, same as Z.
38w, same as w.
37 to 1, autosomes.

PLATE 1.

FIGS. 1 to 6. Cells from the amnion of males of the Indian runner duck. The Z or sex-chromosome is numbered 38Z in each of the cells. 76 chromosomes present.

FIG. 1. Late prophase. The large chromosomes are shown in the characteristic peripheral position with the smaller ones within the circle.

FIG. 2. An earlier prophase than 1.

FIGS. 3, 5, 6. Cells in metaphase. The stippled line in each case indicates a possible grouping of the chromosomes in maternal and paternal groups. This grouping in each case has been considered with especial reference to the 12 largest chromosomes. The autosomes paired from 37 to 33, according to size.

FIG. 4. An early metaphase. Autosomes paired from 37 to 23.

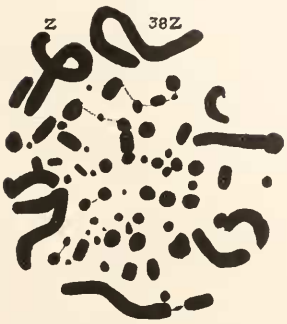


FIG. 1

FIG. 2

FIG. 3

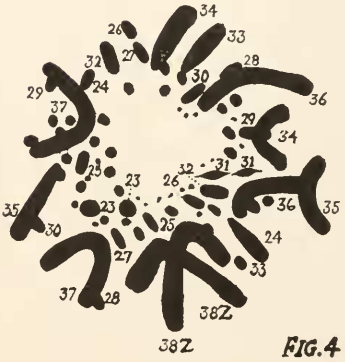


FIG. 4

FIG. 5

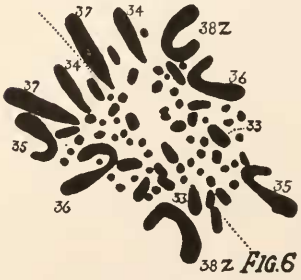


FIG. 6

PLATE 2.

FIG. 7. Metaphase of a cell from the amnion of a male Indian runner duck. The sex-chromosome is numbered 38Z. Autosomes from 37 to 33 are paired according to their size.

FIGS. 8 to 12. Cells from amnion of females of Indian runner duck. W and 38w are the chromosomes that are supposed to carry female-tendency factors only. Some filamentous linkage among the smaller chromosomes is shown in each cell. Autosomal pairs, numbered from 37 down, indicate their relative size in decreasing series. 77 chromosomes present.

FIG. 8. Prophase. Autosomes numbered from 37 to 33. Autosomes 29 are here designated *cr* to indicate their crescent form.

FIG. 9. Late prophase. Autosomal pairs from 37 to 1 indicated. The two groups of five small chromosomes each are shown at the upper left side of the cell.

FIG. 10. Prophase. Autosomes from 37 to 33 numbered.

FIG. 11. Prophase. The place of sutures in No. 37 is well indicated by bendings of the chromosomes in this cell. Autosomes from 37 to 33 numbered.

FIG. 12. Prophase. W in this cell is somewhat foreshortened.

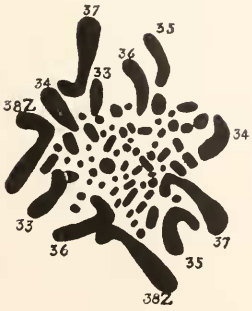


FIG. 7

FIG. 8

FIG 9

FIG 10

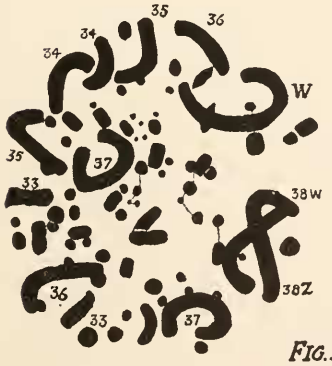


FIG. 11

FIG. 12

PLATE 3.

FIGS. 13 to 18. Cells from the amnion of females.

FIG. 13. Prophase. Autosomes from 37 to 33 numbered.

FIG. 14. Prophase. This cell is atypical in the arrangement of the large chromosomes. However, 77 chromosomes are present.

FIG. 15. Prophase. Autosomes 37 to 33 paired.

FIG. 16. Prophase. Autosomes from 37 to 1.

FIG. 17. An early metaphase. The W chromosome is bent upon itself.

FIG. 18. A late metaphase showing especially gonomeric grouping. The most of the large chromosomes in this cell are foreshortened.

FIG. 13

FIG. 14

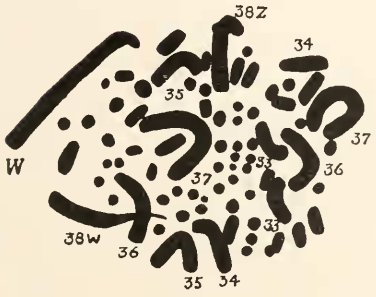


FIG. 15

FIG. 16

FIG. 17

FIG. 18

PLATE 4.

FIGS. 19 to 22. Cells from the amnion of females showing gonomeric grouping of the chromosomes.

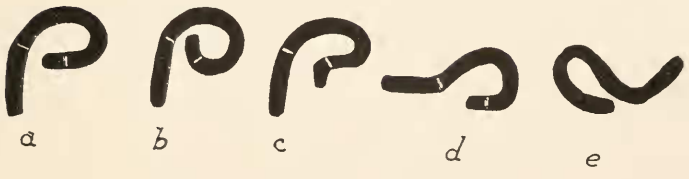
FIG. 23. This figure shows some of the forms assumed by chromosome number 37; also the location of the sutures. Figures a, b, c, d are from cells of the male, and figures e, f, g, h, i are from cells of the female.

FIG. 19

FIG. 20

FIG. 21

FIG. 22



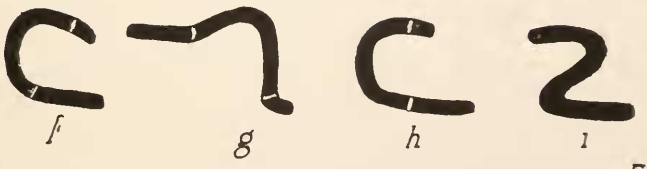


FIG. 23

PLATE 5.

FIGS. 24 to 29. First spermatocytes from smear preparations. The Z-chromosome bivalent in each cell is numbered 38Z, the autosomal bivalents from 37 to 1 according to their size. The cells are in the prophase stage.

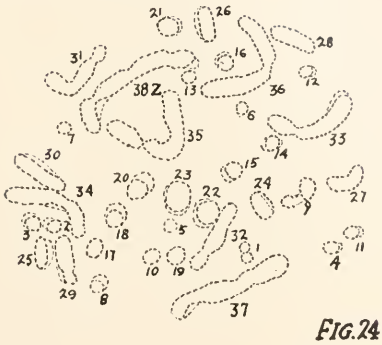


FIG. 24

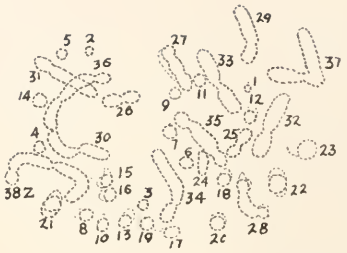


FIG. 25

FIG. 26

FIG. 27

FIG. 28

FIG. 29

PLATE 6.

FIG. 30. Chromosome complex taken from embryonic female gonad of chick. After Hance. In the original drawing the large chromosome at the top of the cell is labeled A to indicate that it is the largest in the complex. It is not paired in the cells of the female. I have added the line to the drawing to indicate a possible gonomeric grouping.

FIG. 31. Chromosome complex taken from a cell in an embryonic male gonad of the chick. After Hance. In the original drawing the two largest chromosomes at the top of the cell are labeled A, a, to indicate that they are the largest in the cell. They are thus present in d uplicate in the male. I have added the line to indicate a possible gonomeric grouping.

FIG. 32. Somatic cell from the female of domestic chicken showing similar grouping. After Schiwago. The lettering is according to Schiwago and is his indication of the "paternal" (right) and "maternal" (left) grouping of the chromosomes.

FIG. 33. Somatic cell from the male of domestic chicken showing same. After Schiwago. The indications are as in figure 31.

FIG. 34. Spermatogonia cell of domestic chicken. After Stevens. The 12 large chromosomes are arranged in peripheral position. I have drawn no line here to indicate gonomeric grouping but such grouping is obvious.

FIGS. 35 to 36. Somatic cell from embryo of lizard (*Sceloporus spinosus*). After Painter. I have again added lines to each of the drawings to indicate possible gonomeric grouping.

FIG. 30

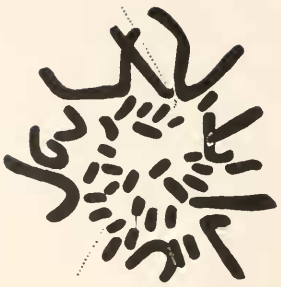


FIG. 31

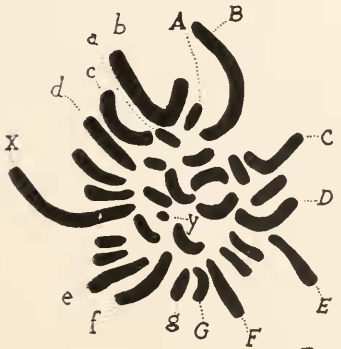


FIG. 32

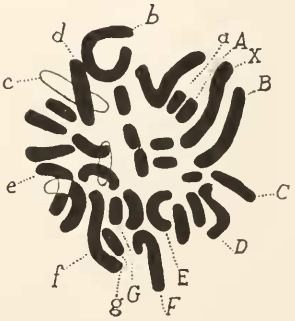


FIG. 33

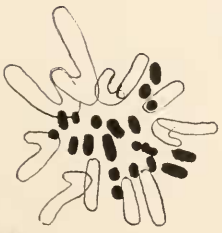


FIG. 34

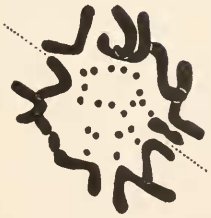


FIG. 35

FIG. 36

PLATE 7.

FIGS. 37 to 42. Chromosomes from the amnion of females artificially arranged according to size. W, w indicates chromosomes carrying female-tendency genes only. 38z indicates the chromosomes carrying sex-linked factors and supposedly a preponderance of male-tendency genes.

FIG. 37. Chromosomes from a prophase shown at Fig. 8. Autosomes 32 are here crescent-shaped.

FIG. 38. Chromosomes from a late prophase shown at Fig. 9.

FIG. 39. Chromosomes from a prophase shown at Fig. 13.

FIG. 40. Chromosomes from a prophase shown at Fig. 11.

FIG. 41. Chromosomes from a prophase shown at Fig. 12.

FIG. 42. Chromosomes from a prophase shown at Fig. 16.

FIG. 37

FIG. 38

FIG. 39

FIG. 40

FIG. 41

FIG. 42

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PLATE 8.

FIGS. 43 to 48. Chromosomes from the amnion cells of males artificially arranged in pairs according to their size. 38Z, 38Z indicate what are probably the sex-chromosomes. Numbers 37 to 1 are probably autosomes.

FIG. 43. Chromosomes from an early metaphase shown at Fig. 4.

FIG. 44. Chromosomes from an earlier metaphase shown at Fig. 7.

FIG. 45. Chromosomes from a metaphase shown at Fig. 3.

FIG. 46. Chromosomes from a prophase shown at Fig. 1.

FIG. 47. Chromosomes from a late metaphase shown at Fig. 6.

FIG. 48. Chromosomes from an earlier prophase shown at Fig. 2.

38Z 37 36 35 34 33 32 31 30 29 28 27 26 25 24 23 22 21 20 19 18 17 16 15 14 13 12 11 10 9 8 7 6 5 4 3 2 1

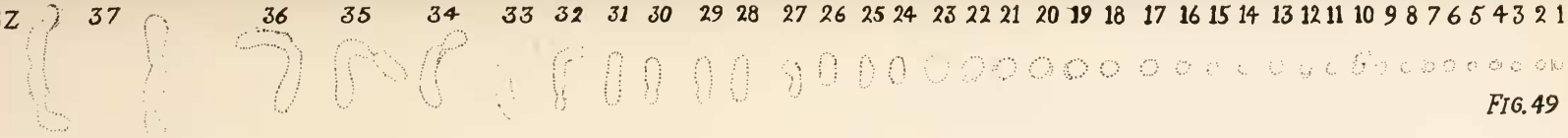

FIG. 49

FIG. 50

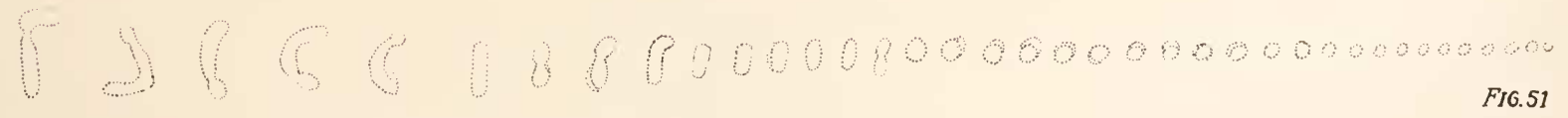

FIG. 51

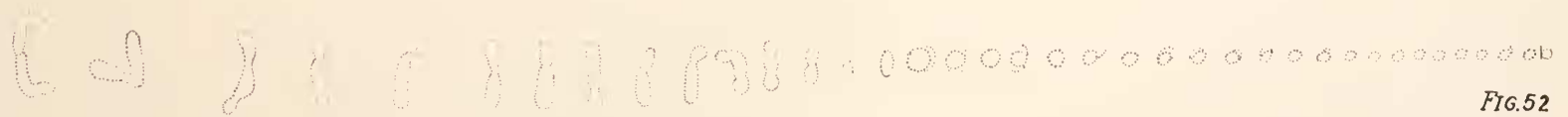

FIG. 52

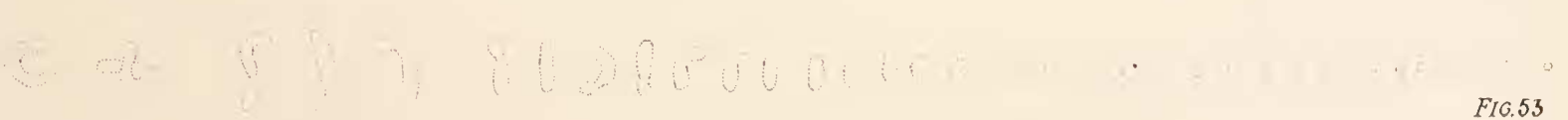

FIG. 53

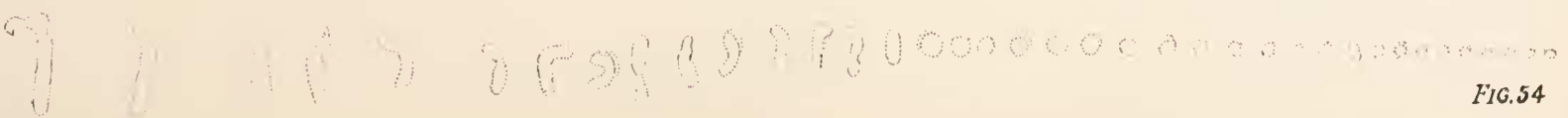

FIG. 54

PLATE 9.

FIGS. 49 to 54. Chromosomes from first spermatocytes from smear preparations. The Z bivalent chromosome is numbered 38Z. The autosomal bivalents from 37 to 1. All the cells are in the prophase stage.

FIG. 49. Chromosomes from cell shown at Fig. 24.

FIG. 50. Chromosomes from cell shown at Fig. 27.

FIG. 51. Chromosomes from a cell shown at Fig. 29.

FIG. 52. Chromosomes from a cell shown at Fig. 25.

FIG. 53. Chromosomes from a cell shown at Fig. 28.

FIG. 54. Chromosomes from a cell shown at Fig. 26.

38Z 38Z 37 37 36 36 35 35 34 34 33 33 32 32 31 31 30 29 28 27 26 25 24 23 22 21 20 19 18 17 16 15 14 13 12 11 10 9 8 7 6 5 4 3 2 1

FIG. 43

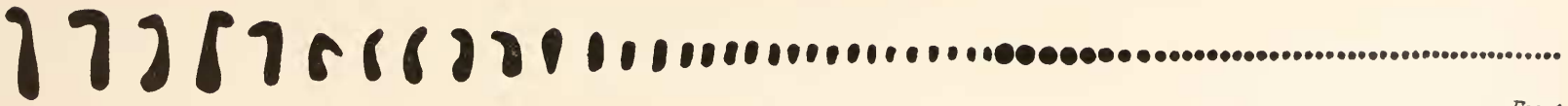

FIG. 44

FIG. 45

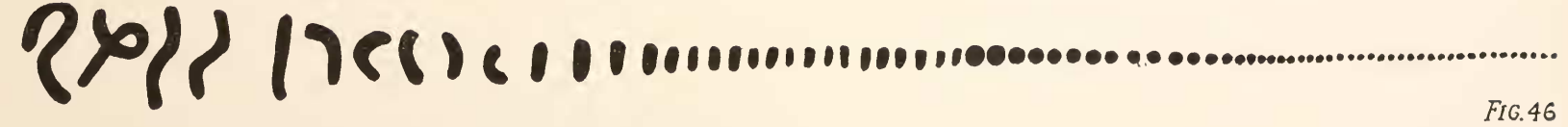

FIG. 46

FIG. 47

FIG. 48

PLATE 10.

FIGS. 35 and 36. Scheme for sex-determination and sex-linkage in the Indian runner duck.

A indicates autosomes.

Z (double lined) indicates sex-chromosome carrying a dominant sex-linked gene, also carrying a preponderance of male-tendency genes.

Z (single lined) indicates sex-chromosome carrying a recessive sex-linked gene, etc.

W indicates the large sex-chromosome carrying female-tendency genes only.

w indicates the smaller sex-chromosome which carries likewise female-tendency genes only.

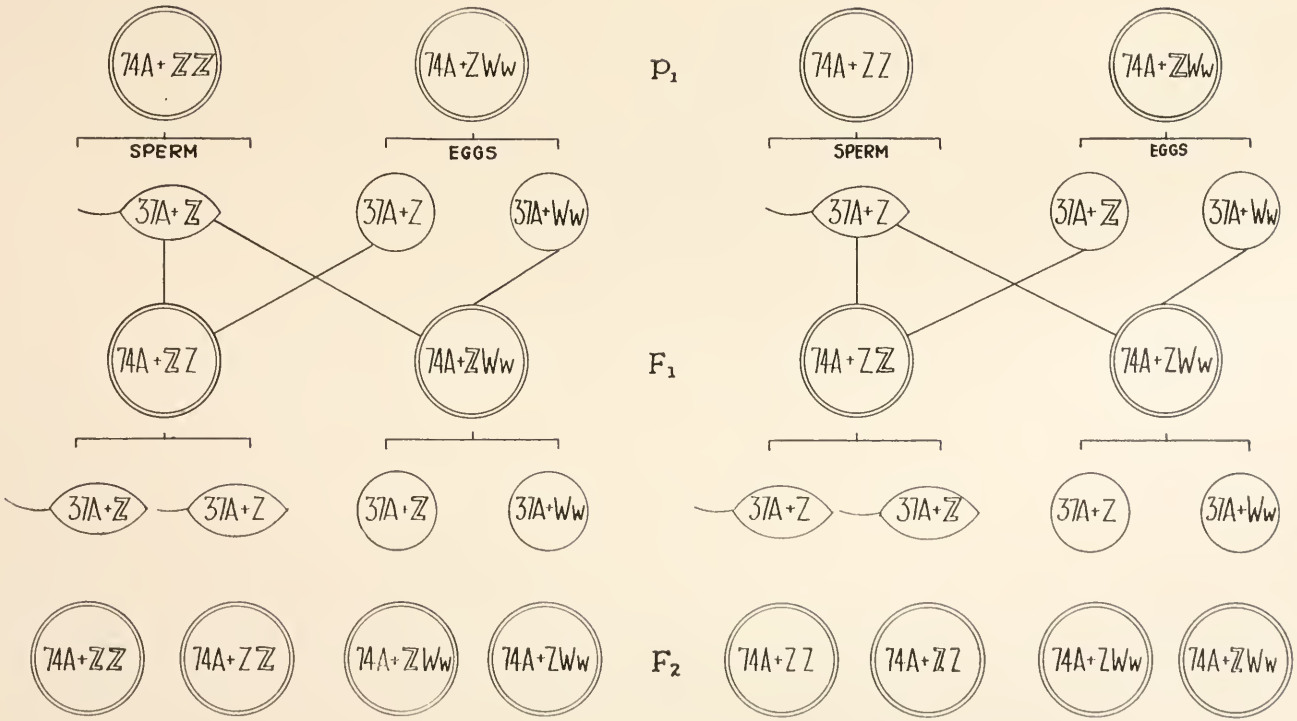

FIG. 56

FIG. 55

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DEFECTIVE *PLUTEUS* LARVÆ FROM ISOLATED BLASTOMERES OF *ARBACIA* AND *ECHINARACHNIUS*.

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ORGANIZATION IN THE SEA URCHIN'S EGG.

Ever since Driesch in 1891 announced that a single blastomere of the two cell stage of the sea-urchin's egg separated by shaking would develop into a whole larva of half size, this method has been generally used by embryologists for determining the type of organization present in various eggs at the time of the first or later cleavages. Driesch's conclusions led to much controversy, but most investigators have come to a fairly definite agreement on the major facts to be learned from this field of investigation, and have turned to other problems. The recent important work of Von Ubisch (1925) has once more drawn attention to the organization of the sea-urchin egg previous to cleavage, and has made it necessary to revise certain accepted ideas. The data which I shall present appear to be of significance in this connection, and even though at this late date it would seem impossible to add anything to the facts regarding the development of isolated blastomeres of sea-urchin eggs, this will be my excuse for briefly reviewing the evidence.

In 1900 and in 1906 Driesch repeated and amplified his earlier work and stated his conclusion that in the sea urchin egg there was no evidence of differentiation at any cleavage stage, and that the egg was an harmonic equi-potential system, each cell of which might give rise to any part of the whole larva. In 1901 Boveri studied the normal development and later isolated blastomeres, and concluded that there was an entoblast zone below the equator of the egg and at right angles to its polar axis (colored red in *Paracentrotus*), without some portion of which an isolated blastomere would not develop an archenteron. He believed that

the first two cleavage planes cut this differentiated zone at right angles, and that the first cleavage plane marked the median plane of the larva. In support of this idea he pointed to the fact brought out by Driesch himself that the isolated animal half of the transversely divided eight cell stage seldom gastrulated, while the isolated basal half (vegetative portion) more often developed normally. Driesch did not accept this interpretation, and in 1906 described the development of an egg incompletely separated in the two cell stage but differentiating as one individual. Here he found that the skeletal rudiments appeared in the descendants of one only of the original cells, and he concluded therefore that the first cleavage plane cut at right angles to the median plane of the larva, and that, contrary to Boveri, the egg was first divided into animal and vegetative portions.

Here the matter has stood up to the present. The majority opinion has accepted Boveri's evidence, as shown by Conklin's summing up of the situation (1924, page 586): "It is plain that there is a differential distribution of egg substances to the cleavage cells from the animal to the vegetative poles, though no differentiation of cells in any cross axis can be detected until much later." E. B. Wilson (1925, page 1067) said: "These facts (*e.g.*, of Boveri) demonstrate that the sea-urchin egg is no more isotropic than that of the mollusk or annelid."

The experiments of Von Ubisch mentioned above prove that both Driesch and Boveri were right with respect to the relation of first cleavage plane and the median plane of the embryo, yet in spite of this are in no sense incompatible with Boveri's idea of a differential organization of the egg at right angles to the initial egg axis. This investigator has succeeded, where Boveri failed, in staining one or more of the blastomeres in dividing eggs of *Echinus* and *Echinocyanus* and then noting the portions of the larvæ which are marked with the dye. By this method he has demonstrated quite conclusively that there is no constant relation between the first cleavage plane and the median plane of the larva, since the stained area may appear in any region of the pluteus. Thus larvæ have been found which differentiated both as described by Driesch and by Boveri, and at every angle between. In spite of this fact Von Ubisch believes that his results

lend support to Boveri's conception that the egg is stratified at right angles to the initial egg axis. With such a distribution of differentiated substances and the first cleavage cutting at any angle, one would expect that staining one blastomere of the two cell stage would give larvæ with the blue area in every possible sector. This Von Ubisch found. Such a result obviously is not at variance with Boveri's view, but it can hardly be called evidence in favor of it for the same might be true if there were no organization of the egg whatever. The only disproof of the latter idea would consist of the demonstration of the same sort of relation in larvæ actually developed from isolated one half blastomeres. This evidence I have secured for *Arbacia* and *Echinarachnius* as will be indicated below.

In addition to the problem of the organization of the undivided egg of the sea urchin with respect to stratification of differentiated substances, there is the added question as to a possible bilateral organization of such materials. Boveri obviously held that the right and left sides of the body of the larva were determined by the plane of the first cleavage, but Von Ubisch's work disproves this. The same criticism applies to the conclusions of Schaxel (1914) and of others who worked with isolated blastomeres. If bilaterality bears no relation to the first cleavage plane, then it must be determined in the egg before the first cleavage or at a much later stage in development. To establish the first relation, since the first cleavage cuts the egg at any angle, it would be necessary to show that isolated blastomeres formed only certain definite parts of some obviously bilateral structure like the skeleton. It seems extremely unlikely, and yet curiously enough there is good evidence for exactly this situation in at least one sea-urchin, *Paracentrotus*—the same egg, by the way, in which appears the stratified pigment layer described by Boveri. J. Runnström (1914) described the development of isolated blastomeres of this egg, and for some reason his results seem to have been largely overlooked in discussions of the subject. Quite contrary to the results of Driesch on *Echinus* and *Sphaerechinus* he found that the blastomeres of the two cell stage did not develop as whole larvæ even after the blastula stage. Instead they gave rise to partial larvæ, showing the skeleton of either the right or

the left side. Such larvæ never become normal. Unfortunately data are not given to show how many blastomeres developed in this manner, nor what happened to the other member of the pair when one gave this result. Apart from this, however, Runnstrom's conclusion seems justified that there was an indication of a bilateral organization of skeleton forming material in the undivided egg of *Paracentrotus*. I have found a number of cases of the same sort in *Arbacia* and *Echinarachnius*, suggesting that in these eggs also there is already some bilaterality of skeleton forming material in the undivided egg.

The bearings of the present investigation may be shown with greater clearness by reference to a diagram. Several of the earlier writers have indicated that material which goes to form the larval skeleton seems to be localized in the four micromeres cut off from the lower quartette of cells at the fourth cleavage (sixteen cell stage). Von Ubisch has apparently confirmed this by staining these cells and finding that the material derived from them formed the larval mesenchyme. On Boveri's view this skeleton forming substance may be thought of as stratified at right angles to the initial egg axis in the undivided egg. That this is probably the case in *Arbacia* is indicated by the recent work of Harnly (1926). He reported experiments in which fertilized eggs of *Arbacia* were cut in two before the first cleavage, and he found that the nucleated portions segmented either as the dorsal, lateral or ventral hemisphere as determined by the presence or absence of micromeres. This result shows, he believes, that there is a localized equatorial area of micromere forming substance in the undivided egg below the nucleus and in the vegetative half of the egg. The later history of these fragments is not described, but we may assume that the material which will form the skeleton is located in the egg as suggested. According to Von Ubisch's evidence the first cleavage plane may cut the egg as shown in Fig. 1. *A* and *C* indicate the extremes, and *B* one of the possible planes between. According to this plan we should expect that each blastomere of *A*, if isolated would develop as a whole larva of one half size. If those of *C* are separated, however, the upper blastomere would lack the skeletal material, and one complete larva and one lacking a skeleton

might be expected. Finally if one of the many eggs in which the cleavage fell between the two extremes, like *B*, was operated on, either the same result or two larvæ with defective skeletons might be looked for. The possibility of bilateral organization

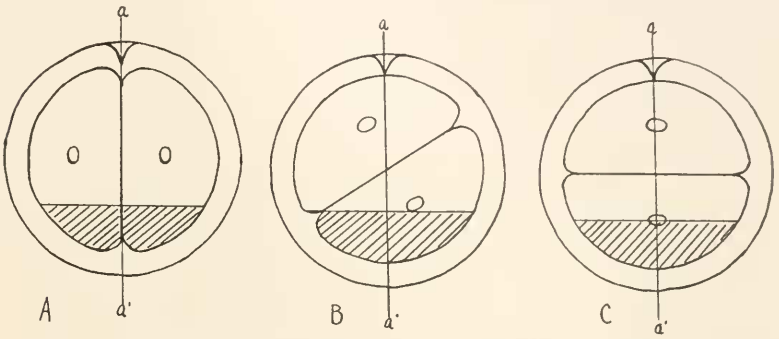

FIG. 1. Diagrams showing three possible relations of the first cleavage plane to the primary egg axis as indicated by the micropyle. The line *a–a'* is drawn through the egg axis in each case. The shaded layer suggests the possible location of the skeleton forming material.

need not be considered for the present. In any case the results of the development of isolated blastomeres, would seem to offer conclusive evidence as to the differentiation in the sea-urchin egg in the light of the work of Von Ubisch.

Considered from this point of view it becomes an important matter to secure data on the later development of a large number of isolated blastomeres of sea-urchin's eggs. Such data must record the complete results and not simply the fact that complete larvæ of one half size are formed from isolated blastomeres of the two cell stage. It is obviously quite as important to know how many such blastomeres do not form complete larvæ, as to know how many do. Practically all of the earlier investigators note that a number of failures occur, but they are content with the positive demonstration. Even under the best conditions and with the greatest care in handling however, a certain number of whole eggs fail to develop normally, and this is sure to be true to an even greater extent when the cells are subjected to the handling required by the technique of separation of the blastomeres. This is true even when the earlier stages of cleavage are normal. Data of the sort suggested therefore can not be

conclusive, for one can never be certain how many of the failures are due to deficiency of materials in the initial cell and how many to abnormal development resulting from the handling.

This difficulty in interpreting the results can be eliminated to a very large degree if the development of both members of the pair of blastomeres from one egg are followed, and the results recorded for single pairs rather than for groups of cells. When one or both of the blastomeres fail to develop to the stage of the ciliated larva, this pair need not be considered. When, under the same conditions and with identical handling, both blastomeres do develop to this stage the result may be considered significant evidence of organization of the undivided egg. It seems an extraordinary fact that in all of the published work on isolated blastomeres of sea-urchins there is so little in which the developmental history of each of the blastomeres derived from a single egg is known. In all of his earlier work Driesch was content to show that whole larvæ of one half size were formed from isolated one half blastomeres, and no attempt was made to prove that both blastomeres from one egg did so, nor even to indicate how many out of a large group failed to differentiate in this way. In 1906 Driesch published his first study of the "Physiology of Bilaterality," in which he did take up this question. He followed the development of both blastomeres of the two cell stage, usually two eggs at a time, in several series of experiments on *Echinus multituberculatus* and *Spœrechinus*. The eggs were observed for two days only, and failures were not recorded. From this data I find that out of 9 eggs divided, only 2 gave normal one half size plutei from both blastomeres, 4 gave two "prismen" larvæ—too young to determine whether the skeleton was normal—2 from which one pluteus was complete and the other apparently incomplete, and 1 in which no skeleton appeared in either. These data are insufficient for any conclusion as to the organization of the egg along the lines indicated, yet so far as they go they suggest that both blastomeres do not necessarily develop into whole larvæ. In all of Driesch's remaining experiments, including several series reported in his 1908 paper on the same subject, the blastomeres were observed for one day only—that is to the gastrula stage—and it was assumed that they would develop into

normal larvæ if they formed normal gastrulae. This assumption is quite unjustified, as my data on *Arbacia* show.

Preliminary Experiments.

Several years ago at the Marine Biological Laboratory I began experiments to determine whether in the egg of *Arbacia* both blastomeres isolated in the two cell stage would form normal half size larvæ. Each egg was handled separately, and the development was followed for at least four days. The work of the first season—1924—gave so few pairs of blastomeres each of which developed that the point could not be settled. At the same time it became apparent that one half blastomeres give rise: (1) to ciliated larvæ which either do not gastrulate or do not form a skeleton, (2) to larvæ with incomplete or partial skeletons, and (3) in a small number of cases to whole larvæ of one half size. These facts I reported briefly at the Washington Meeting of the Society of Zoölogists in 1924.

During the past two seasons at Woods Hole I have been able to extend these observations on *Arbacia*, and during 1926 I made a similar series on the egg of the sand dollar *Echinarachnius*. The latter egg is much more favorable for such work, since it is larger, has a membrane which is much less resistant, and when good lots of eggs are obtained, gives a high percentage of eggs which develop into normal plutei. It is harder to get good lots of eggs of *Echinarachnius* than of *Arbacia* at Woods Hole, and therefore the total number of blastomeres followed is less.

Methods.

The eggs were removed from the animals into sea water, washed, and fertilized with motile sperm suspensions. During the earlier work with *Arbacia*, the membranes were removed by shaking a suspension of eggs in a small test tube for twenty seconds at an interval of one to one and a half minutes after fertilization. This violent treatment is necessary to break and remove the very tough membrane of *Arbacia*, and it generally breaks up or otherwise injures a large number of the eggs. It was later found possible to remove the membranes with great ease, either about fifteen minutes after fertilization or in the two

cell stage, by sucking them up into a very fine capillary pipette with a bore about two thirds the diameter of the egg membrane. If the pipette is of the proper size, and the eggs drawn up carefully by means of an attached rubber tube held in the mouth, this method seldom injures the eggs. It was used throughout the larger part of the work. When the eggs had passed into the two cell stage, a small number were picked up with a slightly larger pipette and placed in a few drops of sea water on a clean glass slide.

The blastomeres were separated with fine glass needles used free hand under a binocular microscope. It is possible to use either one or two such needles, and with practice to acquire a high degree of skill in their use. Needles for this work must be fine enough to lie easily in the furrow between two cells, and stiff enough to allow of a small amount of pressure. Ordinarily needles which are sufficiently fine, work better if they are not too long—about ten times the diameter of the egg seemed to give the most satisfactory results. This free hand method has been used recently by a number of investigators for work in cutting eggs in preference to the more rigid and much slower microdissection apparatus. (Cf. especially Fry 1924, who has given a complete account of the method of making the needles and their use.) It has the advantage that many more eggs can be operated on in a given period, and with practice the control is quite as dependable. The needle is laid along the furrow between the blastomeres, and drawn gently back and forth. Usually *Arbacia* blastomeres can be separated cleanly and without injury by one or two strokes. Often the egg sticks to the needle, and rolls with it, but if this is not prolonged it seldom results in injury. In some cases the blastomeres will separate the width of the needle, but no further, remaining attached apparently by a thin bridge of protoplasm. Such eggs were usually discarded, although the blastomeres seldom come in contact again and eventually separate.

The two cell stage of *Arbacia* with the membrane removed exhibits certain differences from the normal which are of importance in separating the blastomeres. Shortly after division the two cells round out, forming two nearly perfect spheres having a very narrow area of contact. They remain in this condition

for fifteen or twenty minutes, gradually flattening against each other as the next division approaches, until at the time of the second cleavage they appear as half spheres with their flattened diameters in contact. The same process occurs at each cleavage, and is apparently caused by the gradual recurrent increase in viscosity up to the time of actual division. The blastomeres must be separated during the earlier period when they are sphere shaped, since it becomes increasingly difficult later.

I have made many attempts to separate the blastomeres of Arbacia by the use of Ca-free sea water, so widely used for this purpose since the original discovery of the method by Herbst but all of these have been unsuccessful. The original Herbst solution used at Naples, sea water treated with Na-citrate, and various artificial sea waters without Ca, have uniformly failed to bring about the separation of *Arbacia* blastomeres without shaking, and this makes it impossible to keep the two blastomeres of one egg under observation. With *Echinarachnius* the method seems to be somewhat more successful, but not uniformly enough to be superior to mechanical separation.

The isolated blastomeres were picked up one by one with a capillary lip pipette, and the pairs from any one egg placed together in sea water in a round bottomed glass dish two or three centimeters in diameter with a slot ground in the center (Lefevre dishes). These dishes were more satisfactory than depression slides or other containers, since by placing the blastomeres in the slot the water can be changed without danger of sucking up the cells. The development was observed under the four millimeter objective of the compound microscope with a ten ocular.

The dishes when not under observation were kept in a moist chamber consisting of a glass evaporating dish with a cover. This was kept in the tank with running sea water constantly passing over it to maintain it at approximately the temperature of the sea water itself. The water in each dish was changed daily, actual tests showing that a negligible change in hydrogen ion concentration occurred during this period.

In general the cleavage stages of all separated blastomeres were followed and sketched. After that the embryos were observed at least once a day up to the fifth day. Several control eggs with

the membranes removed were placed in similar dishes with each series of experiments.

Experiments.

Summary of All Isolated Blastomeres.—In Table I. a tabular view of all of the results is given, regardless of whether or not both members of the pair of isolated blastomeres developed. All blastomeres which began cleavage normally are included. In the last three columns the figures in brackets are the percentages of the number which lived to the ciliated larva stage.

Table I.

(1) Number of Blastomeres Isolated.	(2) Total Number of Gastrulæ.	(3) Ciliated Larvæ with No Skeleton.	(4) Pluteus with Defective Skeleton.	(5) Complete Pluteus of ½ Size.
Arbacia, 268	121	46 (47%)	24 (24%)	28 (29%)
Echinarachnius 33	20	5 (31%)	6 (38)%	5 (31%)

The summary indicates that a large number of isolated blastomeres even when apparently uninjured, do not develop normally. Less than 50 per cent. of the *Arbacia* cells developed to the gastrula stage, although the number in the sand dollar was larger. Of the isolated cells which lived to become ciliated larvæ,

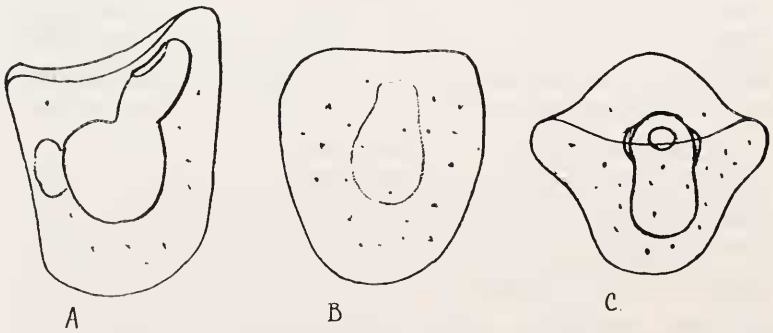

Fig. 2. Ciliated larvæ with no skeletons developed from single blastomeres of the two cell stage of *Arbacia*. Four days old. Measured and drawn free hand from living specimens to the same scale.

47 per cent. in *Arbacia* and 31 per cent. in the sand dollar never developed a skeleton. Doubtless a certain number of these were

injured and therefore failed of normal development, but in view of the evidence given in the next table where the development of pairs from the same egg are listed, it is probable that some of them were deficient in cytoplasmic material already localized at

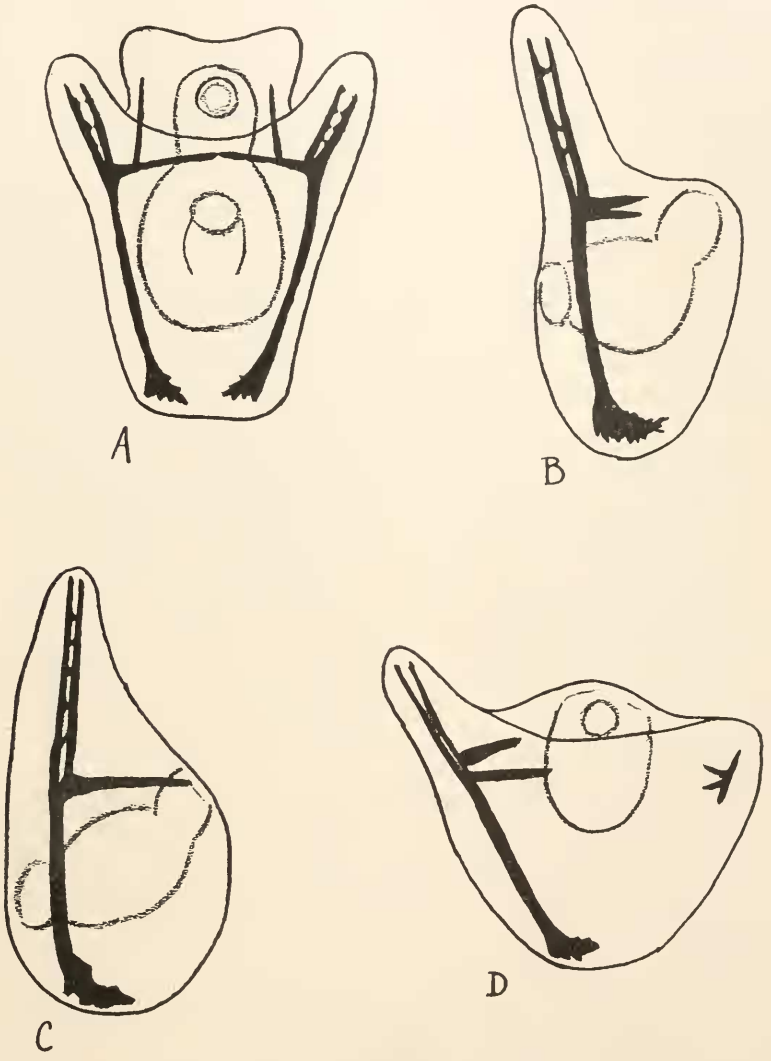

FIG. 3. Pluteus larvæ four days old developed from single blastomeres of the two cell stage of Arbacia. *A* is a larva with a complete skeleton of approximately one half size. *B*, *C*, and *D* are larvæ with defective skeletons. Measured and drawn free hand to the same scale from living specimens.

the time of the operation which later goes to form the skeleton. Many of these larvæ lived for eight days or even more, but always remained enlarged ball-like larvæ (Fig. 2). In perhaps half of these larvæ a normal tripartite gut was formed, while in the others either a small portion or none at all was differentiated. Some of these larvæ were probably derived from blastomeres like the upper one in Fig. 1 *C*.

The larvæ listed in the fourth column of Table I. are of special interest. They were briefly described in my preliminary report. All showed the development of a skeleton but this was incomplete. The commonest type was a four day pluteus with one long postoral arm and the other absent. The oral portion of the skeleton was usually missing entirely. Fig. 3 shows several such larvæ compared with a complete one half size larva of the same stage. Such larvæ of *Paracentrotus* have been figured by Runnström (1914) in earlier stages. It seems impossible to escape the impression that such larvæ are derived from one of the blastomeres of an egg divided like *B* in Fig. 1.

The larvæ listed in the last column of Table I. were normal but of approximately one half size (Fig. 3). They were complete in every observable detail, and were usually as viable as normal control plutei. Such larvæ are probably derived from cells divided along the egg axis (Fig. 1 *A*). It was observed that isolated blastomeres developed at a slower rate than normal control eggs. The first cleavage appeared fifteen to twenty minutes after the second in the normal egg, and the gastrulæ were sometimes two hours or more behind.

Finally it should be stated that I have never found larvæ with defective skeletons among the normal controls unless these were subjected to abnormal treatment. Shaken eggs sometimes give as many as 15 per cent. of abnormal skeletons, but never eggs handled as indicated. Almost every group of control eggs shows a few larvæ which do not gastrulate, or which do not form skeletons, but these seldom live more than two days and usually less.

Pairs of Blastomeres from the Same Egg.

If the results summarized above seem inconclusive because of the large number of blastomeres which fail to develop to the

pluteus stage, it seems possible to attain to a fair degree of certainty by limiting our consideration to those eggs only of which both blastomeres developed to the stage of a ciliated larva. These data I have tabulated in Table II.

Table II.

Showing Types of Larvæ Resulting from the Two Blastomeres of the Same Egg, when Both Live to the Ciliated Stage.

(1) Number of eggs Separated	Pairs of Separated Blastomeres Gave rise to					
	(2)	(3)	(4)	(5)	(6)	(7)
	2 Ciliated Larvæ with No Skeleton.	1 Larva No skeleton and 1 Complete Pluteus.	1 Larva No skeleton 1 Defective Pluteus.	2 Defective Plutei.	1 Defective Pluteus, 1 Complete Pluteus.	2 Complete Plutei.
Arbacia, 18 (100 %)	8 (44%)	5 (28%)	2 | 1 = 17%		1 | 1 = 11%	
Echinarachnius, 9 (100%)	1 (11%)	3 (33%)	1 | 3 = 45%		1 | 0 = 11%	
Representing probable relation of plane of first cleavage to egg axis as in:	?	Figure 1 C	Figure 1 B		Figure 1 A	

Table II. is largely self-explanatory. The total number of eggs of which both blastomeres developed is only 7 per cent. of the total number of blastomeres followed in *Arbacia*, but is 54 per cent. in the sand dollar. When good eggs of the sand dollar can be secured it is obviously a much more favorable species for such work. The same fact appears in column (2), since in 44 per cent. of the eggs of *Arbacia* which gave rise to two larvæ no skeleton developed in either, while only one out of nine, or 11 per cent. gave this result in the sand dollar. This fact introduces a source of error in the interpretation of columns (3) and (4), for it is not certain that some of the larvæ with no skeleton here might not have possessed skeleton forming material. This possible error is not serious, however, as an examination of the remaining data will show.

A study of the remaining columns in the table reveals three fairly striking facts. First, the number of pairs of blastomeres each of which form complete larvæ is very small indeed. I have found only one out of eighteen eggs which gave this result in *Arbacia*, and none out of nine in the sand dollar, although the one recorded in column (6) showed only a slight defect in one arm of one of the larvæ. Second, among such pairs larvæ with defective skeletons are fairly common, but occur most often in pairs, column (5). Probably the error shown by column (2) has prevented some of those listed in column (4) from appearing in (5). Third, there is a fairly large group of pairs of which one member formed one complete half size pluteus and the other a larva with no skeleton, column (3).

For the purpose of our analysis then there would appear to be three significant groups of eggs. While the actual numbers are small they form a selected group from a very much larger number, which as shown above tends to suggest the same result. The first group consists of the small number of eggs each of whose blastomeres formed perfect plutei, the second of eggs each blastomere of which formed an incomplete pluteus, and the third of eggs one blastomere of which formed a perfect pluteus and the other a larva with no skeleton. It is obvious that these three main groups correspond in general with the three possible directions of the first cleavage plane with reference to the initial egg axis as suggested by the work of Von Ubisch, and as indicated in diagrams *A*, *B*, and *C* in Fig. 1. This interpretation is noted in the last line of Table II. Not only are these three groups found, but in the sand dollar eggs at least the percentages of eggs which fall in them correspond with expectation. The second group should be the largest, and the first the smallest, as in fact they are.

Defective Plutei and the Evidence for Bilaterality of Skeleton-Forming Material.

A study of the larvæ with defective skeletons not only substantiates the facts stated above, but suggests in addition a certain amount of bilateral organization of the skeleton-forming material which is apparently already localized in the vegetative half of the egg at right angles to the initial egg axis. All of the

larvæ listed in column (5), Table II., showed defective skeletons. Two sets of these—the *Arbacia* pair and one of the pairs from an *Echinarachnius* egg—were larvæ of the one armed type illustrated in Fig. 3. The other two pairs of *Echinarachnius* larvæ showed skeletons which were clearly complementary, that is the one member showed the parts which the other lacked. These two pairs of larvæ are shown at the age of four days in Fig. 4, *A* and

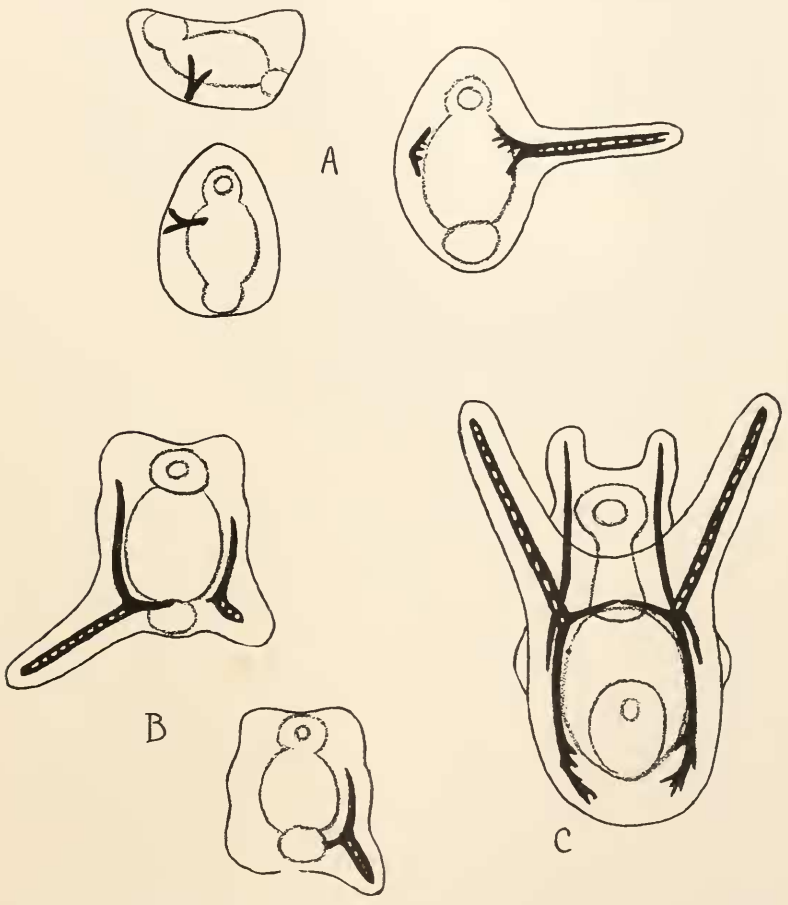

FIG. 4. Pluteus larvæ of *Echinarachnius* four days old. A shows two larvæ developed from the two isolated blastomeres of the two cell stage of the same egg, the smaller one shown from the side and the top, the larger from the top only. *B* shows a similar pair from the top only. *C* shows a normal control larva of the same age, aboral view. Measured and drawn free hand to the same scale from living specimens.

B, compared with a normal control larva of the same age in *C*. All the defective larvæ had a complete tripartite gut like the normal. In the *A* pair one shows a skeleton like the control but with the large *left* postoral arm lacking, while the other has only a rudiment of a spicule on the left side—the *right* side completely lacking. These larvæ were followed for two days longer and the latter never developed any farther, while the former never replaced the defect. In *B* much the same result is shown, perhaps more distinctly, but rather more skeleton appeared in the smaller larva. As indicated above Runnström (1914) described such larvæ in earlier stages as occurring regularly in *Paracentrotus*, and suggested that the undivided egg showed a bilateral organization. The finding of these plutei in *Arbacia* and the sand dollar constitutes very clear proof that a localization of skeleton forming material has taken place in the undivided egg, and that this may be divided unequally by the first cleavage plane. It also indicates a bilateral organization of this material. It seems quite probable that the reason that this bilaterality is not more often evidenced is that blastomeres with a marked deficiency of skeleton-forming material often fail to develop the skeleton rudiment at all.

It may be asked in view of this interpretation how it happens that two complete plutei ever develop from both blastomeres of one egg. To this it may be answered that my data indicate that such cases are extremely rare in *Arbacia* and the sand dollar. When the stratified material is about equally divided apparently the initial bilaterality may be reorganized from the start. That such reorganization may occur infrequently even in later stages seems to be proved by the history of one *Arbacia* blastomere, in which the steps were noted with great clearness.

In the case illustrated in Fig. 5 one isolated blastomere began its development as a partial one, but later a complete but small skeleton was regenerated—or postgenerated. In this case both blastomeres of the egg went through the normal one half cleavage, becoming rounded up at the blastula stage. Two one-half-size gastrulæ were formed, but one never became motile and soon died. The other apparently formed a single triradiate spicule and developed at the end of two days a half skeleton with a single postoral arm, and the crossbars as indicated in the sketch. On

the third day the skeletal material had extended over to the other side and a second postoral arm had appeared. The oral part of

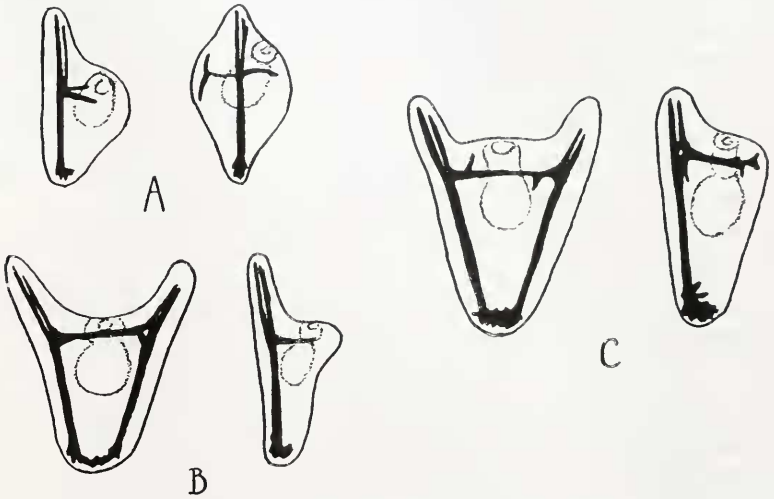

FIG. 5. Successive drawings of a pluteus larva developed from a single blastomere of the two cell stage of *Arbacia*. The skeleton was first one-sided, but later all except the oral portion was regenerated. *A* two days old, *B* three days old, *C* five days old. An aboral and a side view are shown in each case. Measured and drawn free hand to the same scale from the living specimen.

the skeleton was defective. By the fourth day a skeleton with all parts represented, but abnormally massive, and of rather less than the usual size was formed.

CLEAVAGE OF ISOLATED BLASTOMERES.

The work of Driesch, of Morgan, and others indicated that the cleavage of isolated blastomeres of *Echinus* and *Sphærechinus* was that of half embryos. Each blastomere divided into four equal cells, and at what corresponds with the fourth cleavage of the original egg gave rise to two micromeres, two macromeres and four mesomeres, just as they would have done in the normal egg. They later formed an open blastula with half the normal number of cells, which eventually rounded up. Since in the cleavage of *Echinarachnius* there are no clearly distinct micromeres, it is impossible to compare its cleavage with that of the others. The cleavage of the one half blastomeres of *Arbacia* was followed, but

because of the difficulty of being certain of all the cells when rapid cleavage was going on, and when a number of eggs are under observation at the same time, I have accurate records on only about twenty-four blastomeres of this type. Fifteen of these developed as stated above for *Echinus*, namely giving off two micromeres at the third cleavage (normal fourth). The others appeared to have formed no micromeres at all—rather eight equal cells—though two may have had one only. Only eight of the total number developed to the pluteus stage, a number far too small from which to draw positive conclusions. From half eggs showing two micromeres there developed larvæ both with and without a skeleton, and in two cases a larva with a skeleton developed from blastomeres in the cleavage of which no micromeres were observed. So far as they go the data suggest that there is no essential relation between the presence of micromeres in artificially divided eggs and the development of a skeleton. Yet Von Ubisch has shown that the skeletal material is normally passed out into the micromeres, and Harnly's work apparently indicates that when the vegetative portion of the undivided egg is cut off, micromeres do not develop. On the other hand Tennent and Taylor, working on another egg cut before fertilization, did get normal larvæ from fragments which formed no micromeres. It seems probable that normally the pattern of cleavage is determined by the first cleavage plane, and the skeleton forming material goes as usual into the micromeres. When the first two blastomeres are severed this pattern is ordinarily continued, but it may sometimes be disturbed. Whether it is or not apparently has no influence on the development of a skeleton, since that in *Arbacia* at least appears to depend on the presence or absence of skeleton forming material in either or both of the separated blastomeres.

Since this paper was written the more complete account of the work on development of fragments of the unfertilized egg of *Lytechinus* by Taylor, Tennent and Whitaker (1926) has appeared. Their work convincingly proves that in this egg micromere forming substance is not differentiated before fertilization. Whether their disagreement with the findings of Harnly is due to differences in behavior between *Lytechinus* and *Arbacia*, future work

may show. In any case their observations as to the variability in the number of micromeres agree with my own in the development of the severed blastomeres of the two cell stage of *Arbacia*. If the eggs are similar, it would seem to be indicated clearly that the localization of material which later forms the skeleton occurs between fertilization and the cutting through of the first cleavage.

Direct Determination of the Relation of the Egg Axis to the First Cleavage Plane.

The interpretation indicated by the data presented above, namely that there is a stratified layer of skeleton forming material at right angles to the egg axis which may be cut at any angle by the first cleavage and unequally distributed to the first two blastomeres, is obviously susceptible of a direct test. It is possible to determine the egg axis, using the micropyle as a pointer, by the use of a suspension of Chinese ink in the sea water. The angle which the first cleavage plane makes with this axis can then be noted, the membrane removed, and the blastomeres separated. In practice however this is an exceedingly difficult and time-consuming operation. In *Arbacia* at least it is often difficult to make out the micropyle with certainty, and the large number of blastomeres which fail to develop to the larval stage after membrane removal and separation usually make the laborious determination valueless. I have not been able to carry it out in enough cases with *Arbacia* to make the results of any significance. The method was not tried with the eggs of *Echinarachnius*, which appears to be a more favorable species for this work. In future work I hope to make this determination in a larger number of cases.

The relation of the egg axis to skeleton forming or other material localized in the egg might be tested in the same way by Von Ubisch's method also. The primary axis might be determined, and with this actually in view, a sector of the egg at right angles to it might be stained with the purple dye. Von Ubisch has apparently not made this determination, probably because of the difficulties involved.

Summary and Conclusions.

1. The developmental history of more than three hundred blastomeres of *Arbacia* and *Echinarachnius* isolated in the two cell stage and followed individually for at least four days is recorded.

2. About forty per cent. of these blastomeres form ciliated larvæ with a complete tripartite gut, but these larvæ fall in three classes with respect to the skeleton; (*a*) those which never develop a skeleton, (*b*) those in which the skeleton is defective or partial, (*c*) those with a complete pluteus skeleton of one half size.

3. When the pairs of blastomeres from the same egg both of which developed to the larval stage are considered alone, it is found that they fall into three significant groups: (*a*) a very small number of pairs from which developed two plutei with perfect skeletons of one half size, (*b*), a much larger number from which developed two plutei each with a skeleton which was incomplete, (*c*), a group which formed one perfect pluteus and one larva without a skeleton.

4. These facts appear to prove that there is already localized at the time of the first cleavage in the eggs of *Arbacia* and *Echinarachnius* a layer of skeleton forming material at right angles to the primary egg axis, and in the vegetative half of the egg, which may be cut at any angle by the first cleavage plane and unequally distributed to the first two blastomeres.

5. The eggs of these two sea-urchins are thus shown to be (to use the words of Professor Wilson) "no more isotropic than those of the mollusk or annelid." It seems probable that this is true for other sea-urchin eggs as well.

6. In some cases the two incomplete skeletons of a pair of blastomeres from the same egg are complementary. This suggests further a certain amount of bilateral organization of the skeleton forming material already localized in the egg at the time of the first cleavage. This idea receives support from the observations of Runnström on *Paracentrotus*.

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THE NATURAL GROUPING OF THE BREMIDÆ (BOMBIDÆ) WITH SPECIAL REFERENCE TO BIOLOGICAL CHARACTERS.[1]

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In the introductory pages of his rather negativistic treatise on the psychobiology of bumblebees, Wagner ('06), after calling attention to the immense taxonomic importance of biological characters in the case of spiders, cites—by way of contrast—several examples of the disconcerting polymorphism in the coloration and habits of bumblebees and comes to the following pessimistic conclusion:[2] "These are the facts which compel us from the very beginning to give up all hope of finding fixed and unalterable characters in the taxonomy of bumblebees for the elucidation of the biology of these insects, and *vice versa*, of deriving suitable data from their biology for taxonomic purposes."

As we shall see later, this statement of Wagner ('06), as so many other of his assertions (cf. von Buttel-Reepen, '07, '14; Wheeler, '19; and Plath, '23*b*, '24), is based upon superficial observations and is wholly untenable. During the past fifty years, several important attempts, most of which antedate Wagner's ('06) work, have been made to subdivide the Bremidæ into natural groups. These subdivisions have been based either upon (1) coloration, (2) structure, or (3) habits, and in some cases on two or all three of these.

In this paper the writer wishes to discuss in detail those attempts to subdivide the Bremidæ into natural groups which have been based primarily upon biological characters, and to sub-

[1] Contributions from the Entomological Laboratory of the Bussey Institution, Harvard University, No. 247.

[2] "Dies sind die Tatsachen, welche uns von allem Anfange an zwingen, jede Hoffnung darauf aufzugeben, in der Systematik der Hummeln feste und unveränderliche Züge für die Aufklärung der Biologie dieser Insekten zu finden und umgekehrt aus dem biologischen Kriterium geeignete Angaben für die Systematik derselben . . . zu schöpfen."

ject these latter to a critical examination in the light of extensive biological observations on some of our American species. Before doing so, it seems desirable however to review briefly several efforts at grouping which have been made on the basis of (1) coloration and (2) structure.

Grouping on the Basis of Coloration.

The Austrian entomologist von Dalla Torre ('82) is apparently the only one who attempted to subdivide the Bremidæ on the basis of coloration. However, since many species of the genus *Bremus* (*Bombus*) which are not closely related show very striking similarities in coloration, *e.g.*, *Bremus americanorum* [3] and *Bremus terricola*, this method of grouping is a very artificial one and for this reason has not been favorably received by other workers. In this connection the present writer would like to confess that he himself, not long ago (cf. Plath '22*a*, pp. 40–41), made a similar mistake with respect to the genus *Psithyrus*.

Grouping on the Basis of Structure.

There have been several attempts made to group the Bremidæ on the basis of structure. The first of these was by the Russian General Radowszkowski ('84) who divided the genus *Bremus* into eleven groups. This scheme of grouping was later somewhat modified by Franklin ('12/'13) in his "Bombidæ of the New World," in which the then-known, eighty-five American species of the genus *Bremus* are divided into seven groups, a procedure which has been followed by other American workers. This method of grouping has been further extended by Franklin ('12/'13) to the American species of the genus *Psithyrus* which he divides into three groups.

Another scheme of grouping which has much in common with Radowszkowski's ('84) is that of Vogt ('11) who divides the genus *Bremus* into nine subgenera, which Ball ('14) and Krüger ('16 and '20) later increased to ten and thirteen respectively. The last-named author, in addition to making a few modifications in

[3] Dr. Joseph Bequaert and the writer have recently studied De Geer's description and figure of *Bremus pennsylvanicus*, and, like Dr. T. H. Frison ('23), have come to the conclusion that it is better to use the name *americanorum* until the type specimen of De Geer is located.

the nine subgenera established by Vogt ('11), has divided the genus *Bremus* into two sections, on the basis of the presence or absence of a spinous projection on the posterior, distal angle of the metatarsus of the middle legs. This character may prove of considerable importance in determining the genetic relationship of the various species of bumblebees, since, as we shall see later, it seems to go hand in hand with certain fundamental differences in the method by which these insects feed their larvæ.

Another independent subdivision of the non-parasitic bumblebees on the basis of structure is that by Robertson ('03), who removed certain species from the genus *Bremus* (*Bombus*) and erected the genus *Bombias*, chiefly on the basis of the size and position of the ocelli. This innovation does not seem to have found favor with European workers, but *Bombias*, either as genus or subgenus, is in common use in the United States.

Among the attempts to divide the Bremidæ into natural groups on the basis of structure, we may also include that of Friese and von Wagner ('10), who constructed a "Stammbaum" which is intended to show the probable genetic relationship of the fifteen species of *Bremus* occurring in Germany. This scheme of grouping will, no doubt, have to be considerably modified, since it is not in harmony with certain fundamental biological and structural characters which have been pointed out by Sladen ('99 and 12) and Krüger ('16 and '20).

Grouping on the Basis of Habits.

The first one who used biological characters for subdividing the Bremidæ was the English entomologist Smith ('76). In the second edition of his "Catalogue of British Bees," he divides the English species of the genus *Bremus* into Surface-builders (Section I.) and Underground-builders (Section II.). While this method of subdividing the Bremidæ may be of some value to the amateur naturalist, it is of little importance for taxonomic purposes, since certain species of bumblebees, *e.g.*, *Bremus vagans*, make use of many diverse nesting sites: underground, on the surface, in stone walls, hollow trees, birds' nests, attics, fur coats, etc.

A more promising and dependable biological character for

dividing the Bremidæ into natural groups was discovered by the late F. W. L. Sladen ('99) who called attention to the fact that the various English species of the genus *Bremus* employ two very distinct methods of feeding their larvæ. Since this short, but epoch-making paper of Sladen ('99) has been overlooked by several workers, and since it forms the chief basis for the present discussion, it seems advisable to quote the two following paragraphs from it.

"Taking nests of humble-bees and keeping them under observation in specially constructed hives has been a hobby of mine for some years, and it is astonishing what a quantity of interesting information one can gather in this way about the habits and life-history of the dozen or so species that have been recorded from this country, several of which are very common and familiar to every one. It appears that each species has habits and proclivities more or less peculiar to itself, and these, if they could be accurately observed and recorded, would help very much in the systematic arrangement of the species, which in this interesting genus is unusually difficult, owing to the lack of easily recognisable structural differences, and to the little reliance that can be placed on colouring.

"As a result of taking a number of nests it appears that most of the *Bombi* found in this country may be separated into two groups, on what seems to be a rather important difference in the manner of raising their young. These groups may be conveniently named (1) the '*pouch-makers*' and (2) the '*pollen-storers*.' The *pouch-makers* form little pockets or pouches of wax at the side of a wax-covered mass of growing larvæ, into which the workers drop the pellets of pollen direct from their hind tibiæ on the return to the nest from the fields. The *pollen-storers*, on the contrary, store the newly gathered pollen in waxen cells specially made for the purpose, or in old cocoons specially set apart to receive it, from which it is taken and given to the larvæ through the mouths of the nurse-bees as required."

Thirteen years later, Sladen ('12, pp. 40–44, 152–153), in addition to elaborating the foregoing plan in general, changed the term *Pouch-makers* to *Pocket makers*, and further subdivided the latter group into *Pollen-primers* and *Carder-bees*. However,

shortly after this revision had appeared in print, Sladen ('12, pp. 274–275) discovered that the character on the basis of which he had established the *Pollen-primers* is not a dependable one and hence inserted the following paragraph among his "Additional Notes": "*B. latreillellus* was considered to be a pollen-primer because pollen was found under the eggs in a nest in an advanced stage examined in 1911. But in a nest in an earlier stage kept under observation in 1912 the eggs were laid in cells that contained no pollen, although in at least one case pollen was put into the cell and removed before the eggs were laid. Should future investigation show that *latreillellus* is a pollen-primer only under abnormal conditions, a better name for the group, consisting or *ruderatus*, *hortorum*, *latreillellus* and *distinguendus*, would be 'Long-faced Humble-bees'; the term 'pollen-primers' could then be restricted to *ruderatus* and *hortorum*. *Latreillellus* and *distinguendus* are not closely related to *ruderatus* and *hortorum*."

For several summers the present writer has devoted most of his time to the study of the biology of bumblebees, and has had occasion to examine the nests of about 200 *Bremus* colonies belonging to the following North American species: *affinis*, *americanorum*, *bimaculatus*, *fervidus*, *impatiens*, *perplexus*, *occidentalis*, *separatus*, *ternarius*, *terricola*, *and vagans*. This material furnished an excellent opportunity to test the soundness of Sladen's ('99 and '12) classification, as applied to four of our seven American groups. The results of this investigation show that Sladen's ('99 and '12) classification provides an excellent basis for further taxonomic and phylogenetic work, but that certain details of his scheme will have to be modified.

As already stated, Sladen ('12) divides the English Bremidæ into two main groups, the *Pollen-storers* and the *Pocket-makers*. In regard to the last-named group Sladen ('12, p. 44) says: 'When the usual receptacles for pollen employed by a particular species are not available, it may adopt those employed by others. Thus in a strong nest of *B. agrorum*, one of the pocket-making species that I had under observation in 1910, the workers, during a period when there were no growing larvæ and consequently no pockets for pollen, dropped all the pollen they brought home

into a special waxen cell they had constructed, like *terrestris*, on the top of some cocoons. Also a colony of *B. hortorum*, another pocket-maker, being in an advanced stage, and having no growing larvæ, placed pollen in the cocoons vacated by the young queens, but only lined the interior of the cocoons with it."

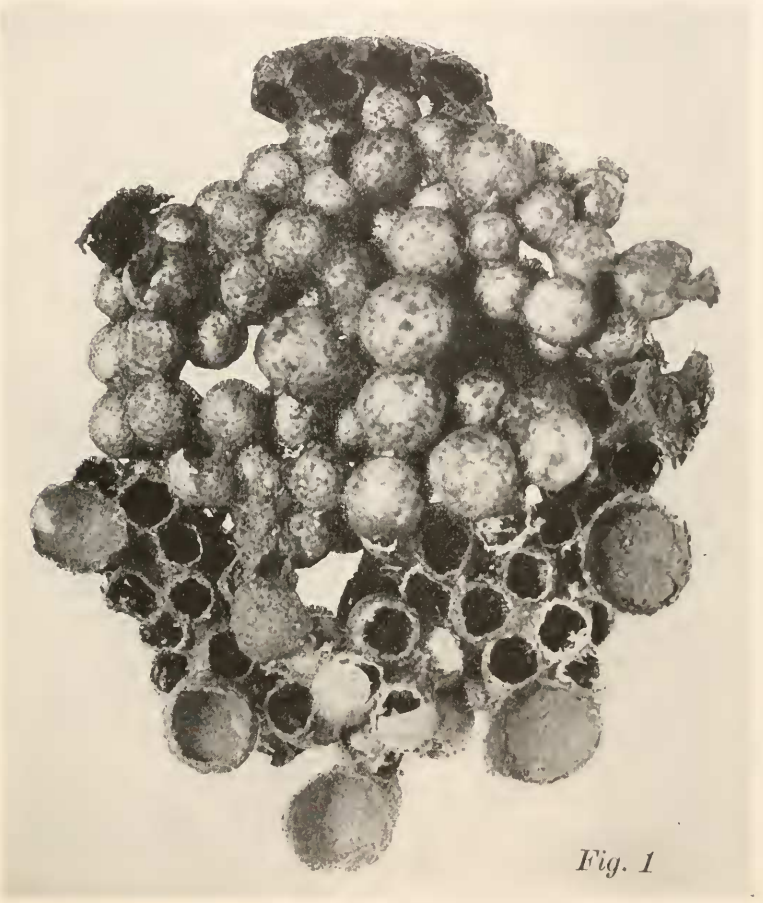

FIG. 1. Comb of *Bremus fervidus* with five pollen bowls on the lower half of the periphery. Natural size.

In this connection the present writer would like to call attention to the fact that *Bremus americanorum* (cf. Franklin, '12/'13 p. 405) and *Bremus fervidus*, two of our American *Pocket-*

makers, not only store pollen under the conditions specified by Sladen ('12, p. 44), but that toward the end of the summer—at the height of brood-rearing—prosperous colonies of these two species store considerable quantities of pollen, in some cases in shallow waxen bowls which are usually constructed near the periphery of the comb. (cf. Fig. 1). From these facts it is evident that Sladen's ('99 and '12) term *Pollen-storers* is of little use in any comprehensive classification of the Bremidæ.

Furthermore, in regard to certain *Pocket-makers*, *e.g.*, *B. americanorum* and *B. fervidus*, it must be pointed out that the habit of making pockets is resorted to only in the case of those larvæ which are destined to become workers, while the brood which furnishes the other two castes (queens and males) is fed by regurgitation; *i.e.*, in the same manner as are those of the non-pocket-making species, a fact to which the writer has called attention in an earlier paper (cf. Plath '23*a*, p. 339). However, since this method of feeding the male and queen larvæ is not employed—at least in the temperate regions—until toward the end of the breeding season, and since the method by which these Bremidæ feed their worker brood is quite distinctive, it seems best to retain the term *Pocket-makers*, unless future investigations show that the use of this term is impracticable.

We now come to Sladen's ('12) division of the *Pocket-makers* into *Pollen-primers* and *Carder-bees*. As already mentioned, Sladen later ('12, pp. 274–275) removed two species from the *Pollen-primers*; but, as will be seen from the following observations, the term *Pollen-primers* will have to be completely rejected as a subdivision of the *Pocket-makers*.

In the fall of 1921 the present writer (cf. Plath '22*a*, p. 34 and '22*b*, p. 195) made detailed observations on a large colony of *Bremus impatiens*, a non-pocket-making species, and in this case the egg-cells were regularly primed with pollen pellets before the workers oviposited in them.[4]

Even more unsatisfactory is the term "Long-faced Humble-bees" which Sladen ('12, p. 275) introduced later, since *Bremus mendax*, which structurally belongs to an entirely different group,

[4] Incidentally this observation also shows that von Buttel-Reepen's ('03, p. 35) explanation of the pollen priming habit of bumblebees is incorrect (cf. also Sladen, '12, pp. 274–275).

and probably is a non-pocket-making species, has a longer face (cf. Krüger, '20, pp. 310 and 359) than either of the two species mentioned by Sladen ('12, p. 275).

Equally impracticable is the term *Carder-bees* which Sladen ('12, pp. 152–153) applies to the second subdivision of the *Pocket-makers*. The collecting of nesting material, the character on which Sladen ('12, p. 17) based this group, is more or less common to all species of the genus *Bremus*, including the non-pocket-making species. However, by the rejection of the term *Carder-bees*, the writer does not wish to imply that the species which Sladen ('12, p. 152) includes under this term do not constitute a distinct group.

Having pointed out the inadequacy of Sladen's ('12) classification as applied to some of our North American Bremidæ, the writer would suggest the following changes in Sladen's ('12) scheme. The name *Marsipœa* [5] (from Greek *marsipos*, a pouch, and poiein, to make) is proposed as a substitute for Sladen's ('12) term *pocket-makers*. This change would result in a suitable name for Sladen's *pollen-storers* (the non-pocket-making species) which could then be designated as the *Amarsipœa*. For reasons already stated, it is further suggested that the terms *Pollen-primers*, *Long-faced humble-bees*, and *Carder-bees* be droped as subdivisions of the *Marsipœa*.

Concerning two species belonging to the *Amarsipœa*, Sladen ('12, p. 36) says: "The larvæ of *B. terrestris* and *lucorum* do not keep together in a compact mass, but as they begin to grow large each one acquires its own covering of wax, although they do not separate completely; the cocoons, therefore, do not form definite clusters, and are easily detached from one another." And in the next paragraph he continues: "With most of the species the skin of wax that covers each batch of larvæ is to the unaided eye unbroken, but as the larvæ grow, *B. terrestris, lucorum, and latreillellus* leave visible holes in the wax, which, when the larvæ approach full size, become large. The larvæ would now run the risk of falling out of their soft wax covering, which would mean their destruction, for a naked larva is always carried out of the nest; but they avoid this danger by enclosing themselves in a

[5] The writer here wishes to express his thanks to Professors W. G. Aurelio and W. M. Wheeler for their valuable suggestions concerning these terms.

loose web of silk, doing this a day or two before they begin to spin their cocoons."

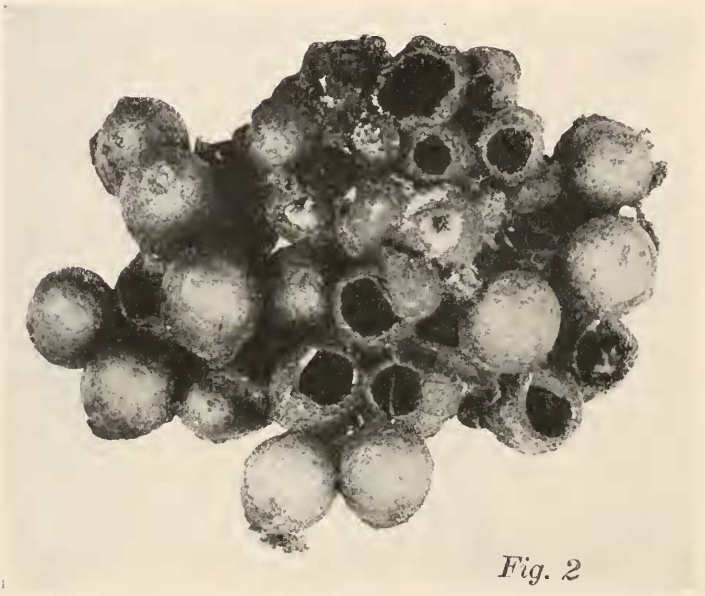

Fig. 2

FIG. 2. Comb of *Bremus affinis* showing exposed larvæ. Natural size.

In this connection the writer would like to call attention to the fact that some of our American *Amarsipœa*, *e.g.*, *Bremus affinis*, *occidentalis*, and *terricola*, have the same habit (cf. Fig. 2). Quite contrary to this treatment of the larvæ is that of the other American and European *Amarsipœa* whose habits have been studied, for the latter carefully keep their larvæ covered with wax (cf. Fig. 3).

Because of this difference in habit, the present writer proposes the name *Phaneroschadonenta* (from Greek *phaneros*, visible, and *schadon*, a bee larva) for those *Amarsipœa* whose larvæ are visible during the greater part of their development, and the name *Cryptoschadonenta* (from Greek *cryptos*, hidden) for those *Amarsipœa* which keep their larvæ covered with wax.

In addition to the very loosely connected cocoons and the uncovered larvæ, the *Phaneroschadonenta* exhibit a number of other peculiarities which mark them as a distinct group. Among

these may be mentioned (1) the short antennæ of the males, (2) the similarity in form between males and workers, and (3) the extreme shortness of the head.[6] This last character is coupled

FIG. 3. Comb of *Bremus impatiens*, a typical example of the *Cryptoschadonenta*. Natural size.

with a short tongue, which makes it necessary for this group of bumblebees to perforate long-tubed flowers in order to secure the nectar. Because of this habit they have been given the name *Disteleologists* (cf. Haeckel, '66; Müller, '79; and von Buttel-Reepen, '14), a subject which the writer intends to discuss in another paper.

The *Phaneroschadonenta* also have the following interesting characteristics: (1) they construct—usually near the center of the comb—one or more bulky, waxen tubes in which they store large quantities of pollen (cf. Figs. 4 and 5); (2) the wax which they produce is unusually dark and brittle, as if mixed with black soil; and (3) their males, like those of the genus *Psithyrus*, are rather sluggish as compared with those of other species.[7] The writer would here also like to point out that the *Phaneroschadonenta* appear early in the spring, that they do not nest on

[6] Cf. Radowszkowski ('84), Friese and von Wagner ('10), Vogt ('11), Franklin ('12/'13), and Krüger ('20).

[7] Cf. Schmiedeknecht ('78), Hoffer ('82, '82/'83), Saunders ('09), Sladen ('12), and Frison ('17).

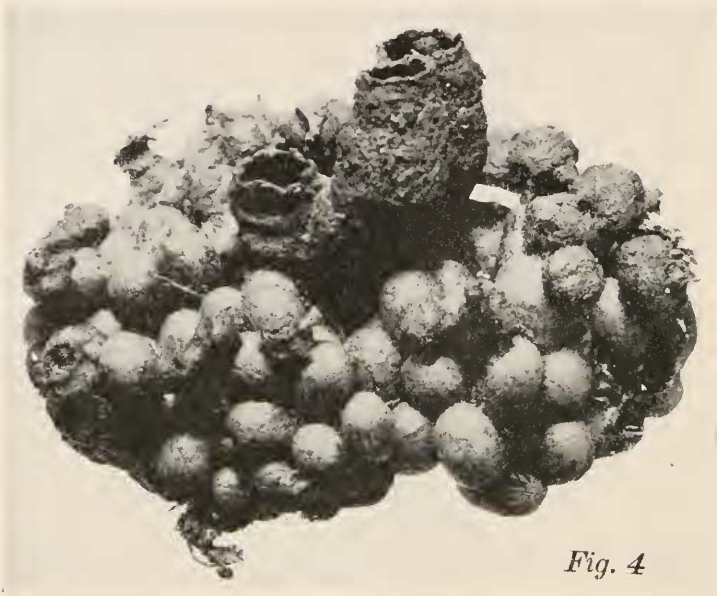

FIG. 4. Comb of *Bremus affinis* showing three pollen cylinders. Natural siz e

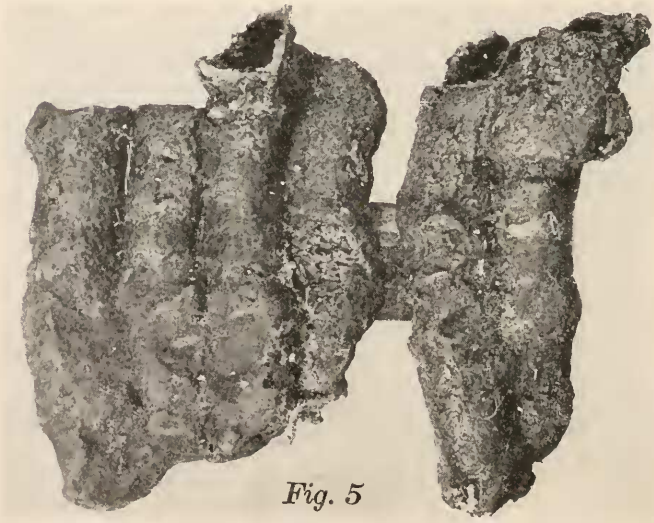

FIG. 5. Six pollen cylinders taken from a nest of *Bremus affinis*. Natural siz Sometimes more than a dozen of these pollen cylinders are found in one nest.

the surface of the ground, and that—as far as known—they do not occur in South America.

However, it may be necessary to use the terms *Phanero-* and *Cryptoschadonenta* only provisionally, since Sladen ('12, pp. 36–37 and 185) states that *Bremus latreillellus*, a *pocket-maker* (*Marsipœa*), also leaves its larvæ uncovered. This seems strange, since the other *Marsipœa* studied are very solicitous to keep their larvæ completely covered with wax (cf. Fig. 6), and this fact

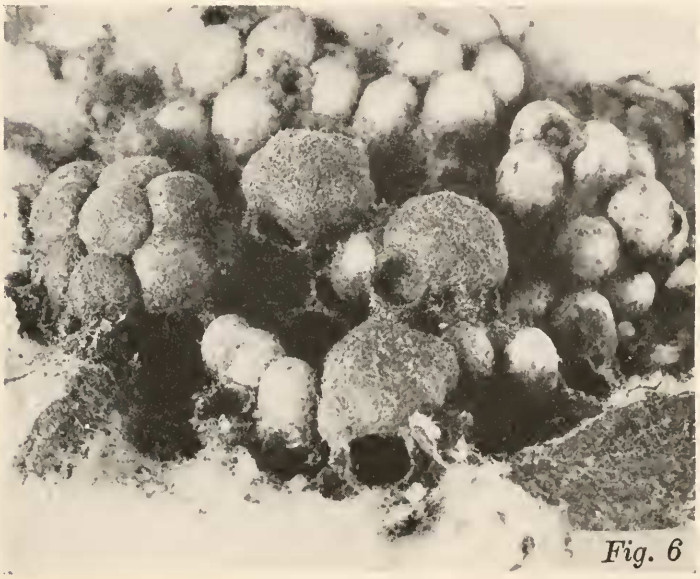

FIG. 6. Comb of *Bremus americanorum* showing (*a*) three pockets, and (*b*) worker larvæ completely enclosed by wax. Natural size.

suggests the possibility that Sladen's ('12) observations on *Bremus latreillellus* may have been made during extremely hot weather, when, due to the softening of the wax, all bumblebee larvæ are likely to become exposed. Should subsequent observations confirm Sladen's ('12, pp. 37 and 185) statement concerning *Bremus latreillellus*, other terms will have to be substituted for *Phanero-* and *Cryptoschadonenta* to bring out the distinctness of this group of bumblebees.

The various American and European species of bumblebees whose methods of rearing their young have been studied up to

the present are listed in Table I. with the suggested modifications of Sladen's ('99 and '12) scheme.

TABLE I.

AMARSIPŒA.

Phaneroschadonenta.

1.	*Bremus affinis*	American Species.
2.	" *occidentalis*	
3.	" *terricola*	
4.	" *lucorum* [8]	European Species
5.	" *terrestris* [8]	

Cryptoschadonenta

6.	*Bremus bimaculatus*	American Species.
7.	" *impatiens*	
8.	" *perplexus*	
9.	" *ternarius*	
10.	" *vagans*	
11.	" *auricomus* [9]	
12.	" *separatus*	
13.	" *jonellus* [8]	European Species.
14.	" *lapidarius* [8]	
15.	" *lapponicus* [8]	
16.	" *pratorum* [8]	

MARSIPŒA.

17.	*Bremus americanorum*	American Species.
18.	" *fervidus*	
19.	" *hortorum* [8]	European Species.
20.	" *ruderatus* [8]	
21.	" *distinguendus* [8]	
22.	" *latreillellus* [8]	
23.	" *agrorum* [8]	
24.	" *derhamellus* [8]	
25.	" *helferanus* [8]	
26.	" *muscorum* [8]	
27.	" *sylvarum* [8]	

Concerning the relative temper of the *Marsi-* and *Amarsipœa*, Sladen ('99, pp. 230–231) says: "It will be noticed that the *pouch-makers* include roughly the timid species. The *pollen-storers*, on the contrary, consist of the bolder species." This is not true of our New England *Marsipœa*, *e.g.*, *Bremus americanorum* and *Bremus fervidus*, these being the most vicious

[8] According to Sladen ('12, pp. 152–153).

[9] According to Frison ('17, pp. 284).

bumblebee species with which the present writer has come in contact, while most of our *Amarsipœa* are comparatively gentle.

It will be interesting to see whether the various bumblebee groups which have been established on the basis of structure are really homogeneous species, when their habits are studied from a taxonomic viewpoint. In the light of our present knowledge, we should expect the seven American groups (cf. Franklin, '12/'13) of the genus *Bremus* to belong to the biological subdivisions as indicated in Table II.

TABLE II.

AMARSIPŒA.

Phaneroschadonenta.

1. *Terrestris* group.

Cryptoschadonenta.

2. *Auricomus* group.[10]
3. *Fraternus* group.[10]
4. *Kirbyellus* group.[11]
5. *Pratorum* group.

MARSIPŒA.

6. *Borealis* group. [11]
7. *Dumoucheli* group.

If Krüger's ('16 and '20) subdivision of the Bremidæ into two sections is really as far-reaching as it appears to be, we should expect the members of the *Borealis* group to belong to the *Marsipœa*, and those of the *Kirbyellus* group to the *Amarsipœa*, since the spine is present in the former, but absent in the latter.

It will also be interesting to investigate the methods which the Bremidæ employ in feeding the larvæ of the genus *Psithyrus*, since certain species of the latter, *e.g.*, *Psithyrus variabilis*, breed in the nests of the *Marsipœa*, while others, *e.g.*, *Psithyrus ashtoni*, are parasitic on the *Amarsipœa*.

Due to a lack of sufficient material, the writer has been unable to determine whether the species for which Robertson ('03) erected the genus *Bombias* have any clearly defined biological peculiarities [12] which would serve as suitable biological characters for distinguishing this group.

[10] Belonging to the genus *Bombias* of Robertson ('03).

[11] Probably.

[12] The males of this group, like those of closely related European species, have their own peculiar habits (cf. Schmiedeknecht, '78; Hoffer, '82/'83; Robertson,

Summary.

1. Contrary to the assertions of Wagner ('06), biological characters are of considerable importance in determining the relationships between the various species of bumblebees, while structural characters frequently make it possible to draw inferences as to the habits of these insects.

2. Our North American *Marsipœa*—at least *Bremus americanorum* and *Bremus fervidus*—feed only their worker brood through pockets, while those larvæ which are destined to become queens and males are fed by regurgitation.

3. The use of the terms *Pollen-primers*, *Carder-bees*, and *Long-faced humble-bees* (cf. Sladen '12) is impracticable in any comprehensive classification of the Bremidæ.

4. Von Buttel-Reepen's ('03) explanation of the pollen-priming habit of bumblebees is not substantiated by the biology of some of our North American species.

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BIOLOGICAL BULLETIN

OBSERVATIONS ON THE LIFE-HISTORY OF *AMŒBA PROTEUS*.[1]

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There is still considerable diversity of opinion concerning the life-history of *Amœba proteus*. Some hold that it reproduces exclusively by binary fission; others do not agree with this.

Carter ('56) asserts that he observed the nucleus in a given specimen, break up into several small nuclei each of which associated itself with a bit of cytoplasm; and that he then saw the membrane of the amœba break and the small nuclei with their surrounding cytoplasm escape into the external medium and move away by amœboid movement.

Wallich ('63), Scheel ('99), Calkins ('05 and '07), Metcalf ('10), Hausman ('20), and Taylor ('24) maintain that they made similar observations. Scheel and Calkins contend that the amœbæ encyst before the nucleus breaks up and Calkins holds that there is a multinucleated generation which ends by sexual activity. Calkins says, "The fertilized cell of Amœba (unknown at present) gives rise to a young amœboid organism formerly known as *Amœba proteus*." Later ('07) he sectioned the amœbæ on which these observations were made and maintains that he found a process of internal fertilization very similar to endomyxis.

Metcalf ('10) asserts that there are two methods of reproduction in which fragmentation occurs; one in which the parent amœba breaks up liberating minute amœboid forms which develop into large amœbæ; another in which small amœboid forms are liberated from the parent by a gemmule formation. These

[1] These observations were made while the authors were working under Dr. S. O. Mast, to whom they are very grateful for timely criticism and helpful suggestions.

amœboid forms he asserts develop flagella, then fuse in pairs, after which the zygotes thus formed develop into large amœbæ.

Taylor gives a detailed description of the nuclear processes accompanying fragmentation. She maintains that the small fragments as they emerge from the parent amœba are cysts which hatch out after varying lengths of time.

All of these observations indicate that *Amœba proteus* at times fragments, forming numerous small amœboid forms, and they appear to indicate that these small forms develop into the large forms which are usually studied. Hausman contends that he actually observed the transformation. There are, however, some prominent investigators, who hold that the evidence presented is not conclusive; for example, Schaeffer, ('26, p. 111.) who says, "We find no proof that the life cycle of the common large ameba includes more than . . . reproduction by fission." The following observations have a definite bearing on this question:

During the last two years we have had under close observation numerous cultures of *Amœba proteus*, started from a few specimens collected in the summer of 1924. During this time it was repeatedly noticed in various cultures that the amœbæ multiplied very rapidly for a while, then suddenly practically disappeared. At first these cultures were all discarded, except a few which were saved for the other protozoa living in them. These were set aside and observed from time to time. After about a week, large numbers of minute amœbæ were found in some of them, and several weeks later numerous large amœbæ.

In further observations it was discovered in several cultures of large amœbæ, (1) that after a period of rapid multiplication by fission the specimens became increasingly more sluggish, darker and more granular in appearance; (2) that they began to decrease in number; and (3) that as the large amœbæ decreased small ones appeared and increased. This did not occur in all cultures, for in some all amœbæ died.

This disappearance of the large amœbæ was observed to occur in several cultures as the solution changed from acid to alkaline in reaction (pH 6.8 to 7.4) or *vice versa*. In other cultures, however, there was no such change, in these the solution remained almost constant as to bacterial content, clearness, amount of food and hydrogen-ion concentration.

Similar results were obtained in cultures on hollow ground slides. All of these cultures consisted of a few drops of filtered culture fluid. One large amœba was put into each of about half of them, and none in the rest. Numerous small amœboid forms appeared in many of the former but in none of the latter. A description of a typical experiment follows:

A large sluggish individual was selected, washed three times in about 5 cc. of distilled water and placed on a hollow ground slide in culture fluid which had been passed through number 50 filter paper. This solution contained no amœbæ that could be seen under a highpower dissecting binocular, or a 1.9 mm. water-immersion objective. The amœba was observed from time to time. The second day after it had been put into the culture fluid on the slide, it became extremely sluggish and remained so. The following morning it had disappeared and in the region where it had been, there were from 150 to 200 minute amœboid forms, about 10 μ in length. A drop of fresh sterile hay infusion was now added every other day for about a week and it could clearly be seen that the small amœboid forms were becoming distinctly larger. Unfortunately, at the end of this time too much solution was added and the forms died.

Following these experiments several amœbæ were isolated and closely watched for a long period of time. The process of fragmentation was actually observed to occur in a number of cases.

The results obtained seem to prove conclusively that *Amœba proteus* at times breaks up into small amœboid forms but they do not prove that these small forms develop into large ones. The evidence presented in the following paragraphs appears, however, to prove this.

On February 19, 1925, two grams of timothy hay were added to 1000 cc. of spring water and boiled for ten minutes. While still hot some of the fluid was poured into a sterile 100 cc. pyrex flask. This was then plugged tightly with cotton. When the flask had cooled a few drops of old culture fluid, which had been passed through number 50 filter paper, were added. The flask was then again plugged with cotton and allowed to stand for a week. Then this culture, free from amœbæ as shown by careful observation, was inoculated by one amœba which had been washed in several

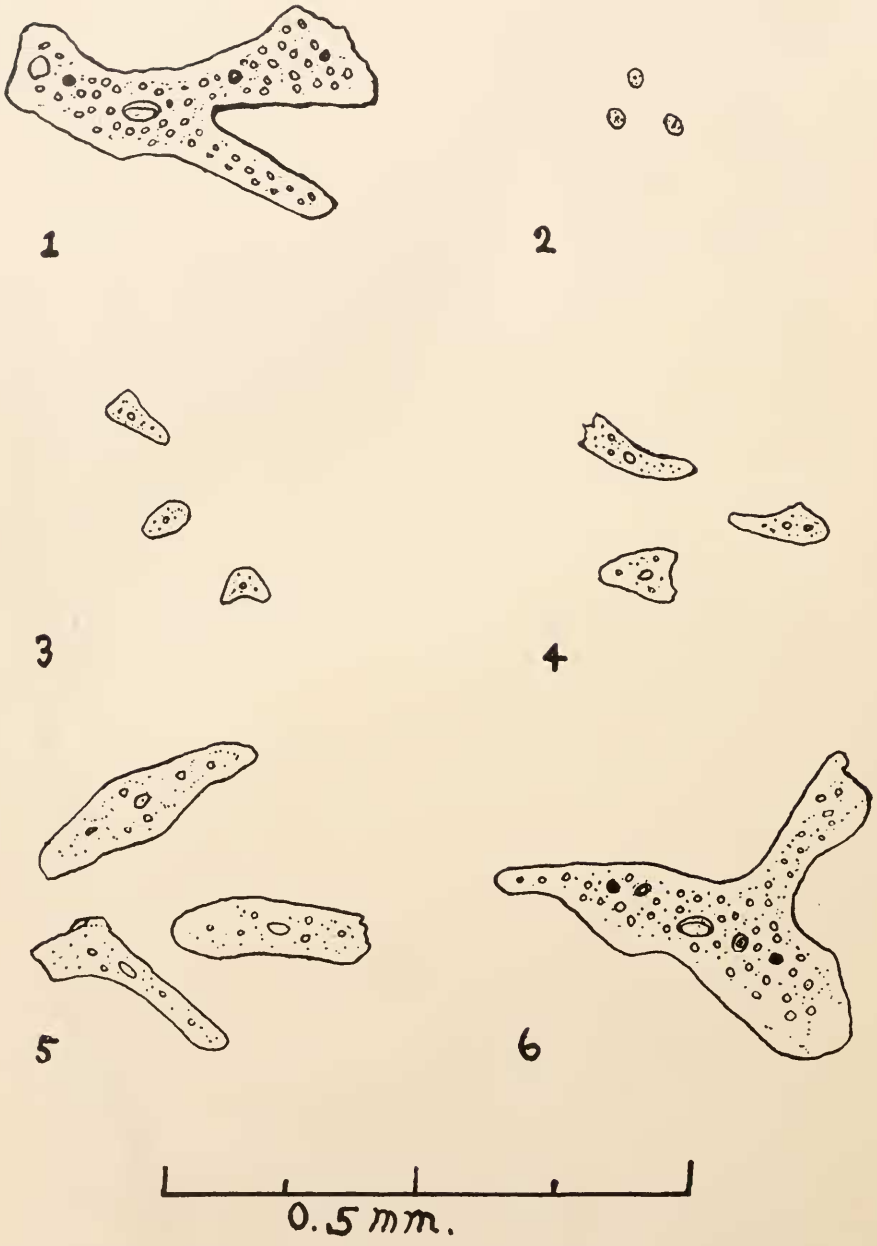

FIG. 1. Camera lucida sketches of some of the largest amœbæ in a culture at different time intervals, showing growth of small amœbæ into large amœbæ. 1, one of the original amœbæ which fragmented, giving rise to small amœbæ; 2,

changes of distilled water. After this there was added each day for food about 5 cc. of fresh sterile culture fluid like the original culture fluid. All pipettes used in handling the amœba and the culture fluid were repeatedly sterilized in order to eliminate contamination.

The culture was poured into a sterile shallow dish and thoroughly examined every few days. It was found that the individual multiplied by fission until in about two weeks there were perhaps fifty large amœbæ in the culture. Then fission appeared to cease and the amœbæ assumed a dark granular color, after which they began rapidly to disappear so that by the end of the third week there were no large amœbæ present. If there had been one large amœba left it could not have escaped the rigid inspection to which the culture was subjected. The culture was now set aside for a week, after which it was again examined. It now contained numerous small amœbæ the length of which varied from 10 to 40 μ, but no large amœbæ. For over a month following this the culture was repeatedly closely observed. The amœbæ increased in size until by the last of April there were numerous specimens which measured 300–600 μ in length.

On September 17, 1926, fourteen cultures made up in a modified Ringer's solution were inoculated with amœbæ from an ordinary spring water culture after being washed in six changes of the Ringer's solution. At the end of four days all of the large amœbæ had disappeared and soon after this small amœboid forms were observed in great numbers. From this time on the cultures were thoroughly examined at the end of each week and after each examination camera lucida sketches were made of the largest amœbæ found in each culture. Some of these sketches are herewith reproduced (Fig. 1). They show that the amœboid forms increased from an approximate average volume of 400 cubic micra to one of 250,000 cubic micra and that this increase required two months. During this time, however, it was observed that these small amœbæ occasionally divided (Fig. 2). All of

small amœbæ produced by fragmentation of original large amœbæ; 3, largest amœbæ in culture ten days after fragmentation; 4, largest amœbæ in culture four weeks after fragmentation; 5, largest amœbæ in culture six weeks after fragmentation; 6, largest amœbæ in culture eight weeks after fragmentation.

these cultures and the one mentioned in the preceding paragraph, were thoroughly examined at least twenty-five times during the two months growth period, and no large amœbæ could be dis-

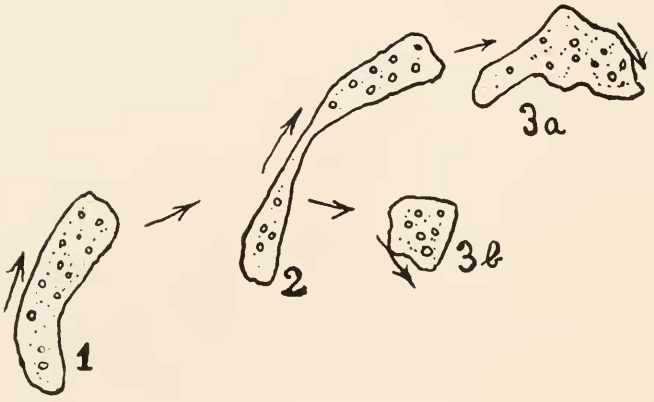

FIG. 2. Camera lucida drawings of a small amœba just before division (1) and (2) and immediately after division (3*a*) and (3*b*).

covered until the end of two months as stated. It is, therefore, evident that even one large amœba could not have remained in the large form unobserved during the growth period of nearly two months.

It is evident that the small forms develop into large ones and that as they do so they divide from time to time.

SUMMARY.

Individuals of *Amœba proteus* sometimes break up into from 100 to 300 amœboid forms. These amœboid forms gradually become larger until at the end of about two months they are as large as the original specimens. During the increase in size division occurs from time to time.

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AN ANALYSIS OF THE SPAWNING HABITS AND SPAWNING STIMULI OF *CUMINGIA TELLINOIDES*.[1]

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Since the discovery of a slight lunar periodicity in the spawning of *Chætopleura apiculata*, the writer has studied the spawning seasons of a considerable number of animals in the hope of finding other more clearly marked cases. The conclusion of this study is that the phenomenon is of rare occurrence at Woods Hole. *Cumingia tellinoides* is the one species studied critically to date that shows a lunar periodicity. Three other species whose study has been completed show absolutely no periodicity. Others being studied require further investigation before they can be passed upon with any degree of certainty. If semi-scientific reports are trustworthy, this type of periodicity is much more common in the tropics, since several fairly well authenticated cases are reported from the Tortugas and elsewhere.

A part of the data presented in this paper was read before the American Society of Zoölogists at their annual meeting in December, 1922. Since that time the study has been continued and the results are finally presented for publication in their completed form.

SECTION I.

THE SPAWNING SEASON.

The Duration and Characteristics of the Spawning Season.

Cumingia begins to spawn about the middle of June and continues with variations of activity until the middle of September and sometimes until the first of October. During 1926 the first eggs were obtained on June 21 and the last ones on September 21. The heaviest spawning occurs during the last

[1] From the Osborn Zoölogical Laboratory, Yale University, New Haven, Conn., and the Marine Biological Laboratory, Woods Hole, Mass.

week in June, July and the first half of August, as stated by Morgan (*Jour. Exp. Zoöl.*, 1910). The production of eggs by each female is continuous and covers the entire period. If *Cumingia* which have spawned vigorously in the laboratory are returned to their normal habitat for three weeks or a month, they will again set free large quantities of eggs or sperm showing that the supply is replenished.

Experiment No. 1.—July 7, 1924. A stake was driven off Ram Island and eighteen *Cumingia* were planted at its base. All these had just spawned in abundance. On August 7, seven of these *Cumingia* were again brought to the laboratory and treated as usual to induce spawning. After forty minutes two females had spawned heavily and three males had shed sperm abundantly. One other female spawned after one and one half hours. One of the seven did not spawn. The eggs of all these were fertilized and grew into normal veligers.

Experiment No. 2.—July 6, 1926. Eighteen *Cumingia* that had just spawned heavily were planted off Ram Island. On August 1, nine of these were again brought to the laboratory. Three females spawned actively, three males shed sperm in quantity, three did not spawn.

These two experiments indicate that *Cumingia* spawns more than once in a season and that the production of eggs and sperm is continuous.

There are indications that each female spawns two or three times during the season when left in the natural habitat. This inference is founded upon the facts that the eggs are constantly produced and that spawning seems to be most common at full moon and the days following full moon. Since there is no direct evidence on this point, I do not care to insist upon it, except to refer to the schedule of spawning experiments. (See tables, Section III. on lunar periodicity.) There are clearly marked variations in spawning activity which appear to be associated with phases of the lunar cycle.

Orton 15 has advocated the theory that the duration of the spawning seasons of animals is determined by temperature. According to his conception, spawning by any summer breeding species begins in the spring when the water reaches a certain

temperature and ends in the fall when the water drops below that temperature. It may be in order to say that this theory is true only in a very general sense, since the species that have been studied by me do not agree among themselves in this regard. Thus *Cumingia* began to spawn on June 21, 1926, when the temperature of the water was 60° F. and quit about September 25 when the temperature was 65° F. *Bugula flabella at* began spawning on June 20 in 1926 when the temperature was 60° F. and continued until November 26, when the temperature was 47° F. *Bugula* usually begins to shed its larvæ by June 10 and continues until late in November, which makes the discrepancy in temperature less pronounced, June 10 temperature being approximately 55° F., November 25 temperature 45° F. *Chætopleura* begins to spawn about June 25, temperature 60° to 65° F. and ends early in September, temperature 65° to 70° F.

Spawning is caused at particular times in nature by various specific stimuli and it is not determined by temperature alone.

Orton gives convincing evidence to show that temperature is the chief factor in determining the limits of the spawning seasons of marine invertebrates. He, however, dismisses the factor light with too little emphasis. Although most tropical animals may spawn throughout the long summer in accordance with his theory, the Palolo worm spawns on one or two days only; and the ripening of its gametes is influenced by light according to several investigators. Light plays a part both in the maturing of the gametes and in spawning in several well-known cases. A very striking example is *Dictyota* at Beaufort, N. C., as described by Hoyt. Temperature, no doubt, is the chief factor in determining the spawning season just as it is also the most important factor in growth and all protoplasmic activity.

SECTION II.

Artificial Spawning Stimuli.

I. Shock.

As stated in my paper on *Chætopleura* (Biol. Bull., 1922), certain marine invertebrate animals will spawn when placed in an artificial or unusual environment, stimulated apparently by

shock. As a case in point, if the tube forming annelid Hydroides is removed from its shell and the exposed worm placed in a dish of sea water it will spawn at once, the eggs or sperm seeming to come from every nephridium of the body. This worm will always spawn under these circumstances whether the gametes are fully mature or not. Half-grown eggs are as readily spawned as mature ones. Hydroides will spawn in the spring before any of the eggs are even approximately mature.

In like manner, *Cumingia* spawn readily in the laboratory. It is only necessary to place them in a bowl of sea water and allow them to remain undisturbed for half an hour or forty-five minutes. They usually begin to spawn in half an hour. The gametes are expelled through the dorsal siphon which is extended to a great length. As the eggs or sperm accumulate in the siphon they are thrown out forcibly by sudden whip-like contractions of these organs. During the height of the breeding season it frequently happens that every individual brought to the laboratory spawns. It is not to be supposed that all would have spawned on that particular day if left in their natural environment. There is then something about the treatment which involves digging them from the sand and subjecting them to various unusual disturbances which stimulates spawning. It is safe to conclude that this spawning stimulus is shock as in the case of Hydroides. Spawning takes place just the same when sea water is allowed to flow gently through the dish so that the accumulation of CO_2 is not a factor. It is to be noted that shock is an abnormal stimulus. It may be said again that laboratory experiments are frequently unreliable guides to natural behavior and may lead to misinterpretation if depended upon too implicitly.

Chemical Stimuli.

The question next arises: do the sexes stimulate each other in any way? The question as to whether the females are stimulated to spawn by the presence of the male and vice versa has frequently been discussed in connection with various species. Some very clear cases of the existence of such a chemical stimulus have been discovered. This, for instance, is the case in *Nereis limbata* as described by Lillie and Just. When placed together in a dish

of sea water both sexes are excited at once to remarkable activity, and while swimming excitedly they expel their sexual products with violent contractions. Galtsoff has recently shown that the female oyster will spawn if living oyster sperm is added to the water. This is opposed to the published statements by Nelson who has thought that the oyster spawns on particularly warm days and that general spawning on oyster beds is induced by temperature. I am under the impression that he is correct in this statement but the implied conclusion that temperature is the only spawning stimulus may be misleading. He shows, however, that spawning occurs on the peaks of rising temperatures which indicates that changes in temperature upward cause males to shed and hence general spawning by all nearby oysters results. Spawning by oysters may take place at any temperature between 68° and 85° F. (Churchill and Nelson).

It was found in the case of *Cumingia* that there is no perceptible stimulus from the opposite sex. They appear to spawn quite as readily when isolated as when in the same dish. Experience has shown that the best way to obtain clean eggs in convenient form for study is to wash the animals free from debris and isolate them in small stender dishes half filled with sea water, or enough water to cover the animal. Both males and females will shed their gametes when so isolated. The eggs are thus obtained free from sperm and may be artificially fertilized at will. Drew was the first to use this method and he was under the impression that drying accentuates or constitutes the spawning stimulus. Morgan in 1910 noted that *Cumingia* will spawn when isolated (*Jour. Exp. Zoöl.*, Vol. 9, p. 595). He thinks, however, that the presence of spermatozoa in the water may incite the females to spawn more promptly than they otherwise would. The stimulus in this case, if authenic, might be either chemical or physical. One is likely to gain the same impression by watching the spawning of *Chætopleura* (*Chiton*) because when males and females are placed together in a dish of sea water, the males always shed their products first, and are followed promptly by the females. So far as could be learned, however, spawning by the females occurred as promptly without spermatozoa as with them.

It is the writer's belief that the spermatozoa have no effect. The mechanical shock of removing the animals from their normal situations furnishes all the stimulus that is required to induce spawning. There is no perceptible stimulus from the opposite sex either chemical or physical. This is shown by a series of experiments designed to test the theory. The three described herewith are representative. The method was to collect *Cumingia* in large numbers. Half of them were isolated in small stender dishes and covered with sea water in the usual way. The other half were treated in the same way except that they were all put in a large crystallization dish so that they might receive chemical stimuli from each other if such exist. The time of spawning is shown in the two columns of the tables and they may be readily compared.

Experiment No. I.

August 21. Collected 20 *Cumingia*. Ten were isolated in stender dishes and ten were placed in a common dish. Experiment set at 12:30 P.M.

10 Isolated Individuals Spawned as Follows.	Time Elapsed before Spawning.	10 in Common Dish Spawned as Follows.
1:15 P.M. one female	45 minutes	
1:16 P.M. one male	46 "	
1:20 P.M. one male	50 "	
1:23 P.M. one male	53 "	
	55 "	One male shed 1:25 P.M.
	57 "	One male " 1:27 P.M.
1:29 P.M. one female	59 "	
	60 "	One male " 1:30 P.M.
	61 "	One female " 1:31 P.M.
	67 "	One male " 1:37 P.M.
	68 "	One male " 1:38 P.M.
	70 "	One male " 1:40 P.M.
	71 "	One female " 1:41 P.M.
1:55 P.M. one female	85 "	
2:05 P.M. one female	95 "	

Comment: Three of the isolated individuals and two in the common dish failed to spawn. Comparison shows that those in the common dish did not spawn more promptly than the isolated individuals. It is noteworthy that those in the common dish

spawned close together in point of time. This was also true in Experiment No. 3, but not in No. 2.

Experiment No. 2.

August 27. Collected 34 *Cumingia*. Isolated seventeen in stender dishes and placed seventeen in a common dish. Experiment set at 5:55 P.M.

17 Isolated Individuals Spawned as Follows.	Time Elapsed before Spawning.	17 in Common Dish Spawned as Follows.
	30 minutes	One female at 6:25 P.M.
One male at 6:27 P.M.	32 "	
One male at 6:28 P.M.	33 "	
One male at 6:29 P.M.	34 "	
	35 "	Two males at 6:30 P.M.
	36 "	One male at 6:31 P.M.
One female at 6:32 P.M.	37 "	
	38 "	One male at 6:33 P.M.
	39 "	One male at 6:34 P.M.
	41 "	One female at 6:36 P.M.
One male at 6:37 P.M.	42 "	One male at 6:37 P.M.
One female at 6:37 P.M.	42 "	
One male at 6:39 P.M.	44 "	
	47 "	One male at 6:42 P.M.
	48 "	One male at 6:43 P.M.
One male at 6:44 P.M.	49 "	
One female at 6:45 P.M.	50 "	
Two males at 6:46 P.M.	51 "	One female at 6:46 P.M.
	52 "	One female at 6:47 P.M.
	55 "	One male at 6:50 P.M.
One female at 6:51 P.M.	56 "	
One female at 6:54 P.M.	59 "	

Comment: Five isolated individuals did not spawn within the hour and five of those in the common dish did not spawn. There is no evidence that they receive a chemical stimulus from each other calculated to induce spawning. They spawn as readily when isolated as when in the same water.

Experiment No. 3.

August 28. Collected 24 *Cumingia*. Isolated twelve in stender dishes and placed twelve in a common dish. Experiment set at 1:30 P.M.

12 Isolated Individuals Spawned as Follows.	Time Elapsed before Spawning.	12 Individuals in Common Dish Spawned as Follows.
	2 minutes	One male at 1:32 P.M.
1:48 P.M. one male	18 "	
	25 "	One male at 1:55 P.M.
	27 "	One male at 1:57 P.M.
1:58 P.M. one male	28 "	One male at 1:58 P.M.
1:59 P.M. one male	29 "	One male at 1:59 P.M.
	30 "	One female at 2:00 P.M.
	35 "	One male at 2:05 P.M.
	38 "	One male at 2:08 P.M.
	39 "	One male at 2:09 P.M.
2:10 P.M. one female	40 "	
2:13 P.M. one female	43 "	
2:15 P.M. one male	45 "	
2:16 P.M. one male	46 "	
2:17 P.M. one female	47 "	
2:20 P.M. one female	50 "	
2:22 P.M. one female	52 "	

Comment: Two of the isolated individuals and three in the common dish did not spawn within an hour. As a matter of chance most of the females got into the isolated dishes.

SECTION III.

Natural Spawning Stimuli.

I. Lunar Periodicity.

If *Cumingia* spawns most frequently at the period of the full moon, it is evident that there is some cosmic stimulus which varies with the moon's phases to which spawning is due. The two chief variable quantities are light, and tides, or pressure. There is at present no scientific explanation of lunar periodicity although numerous cases are known.

It has been the writer's chief interest during the past five years to learn whether the phenomenon is rare or of common occurrence among animals at Woods Hole. The data bearing on this subject are given in a short forthcoming paper, but the findings in reference to *Cumingia* are given here in greater detail.

A careful study and comparison of data collected during the past five years shows that there is a lunar periodicity in spawning, although it is not as well marked as in *Nereis limbata* or the Suez sea urchin studied by Fox.

There is no clock-like regularity in the spawning of *Cumingia* in nature. Not all individuals spawn promptly at the full moon. Spawning in fact covers a period of two or three weeks in each month. We know this by the quantities of eggs spawned in the laboratory by any particular lot of sexually mature adults brought in. Some set free a maximum quantity, some a small quantity and some only a few eggs or none at all. The last are considered to have spawned recently. By this indirect reasoning, one learns that there is no one particular day on which general spawning occurs. It is likely that spawning occurs when the gonads and their ducts become filled to capacity and this internal pressure no doubt constitutes a second natural spawning stimulus. If the production of gametes were continuous, and uniform in rate, spawning might occur on any day in the month except for this cosmic stimulus which brings about a more or less marked rhythm. I am of the opinion that there is a rhythm in the rate of production of the gametes as well as in the spawning of them. In any case, the only time in the month during which most of the gonads seem empty and during which spawning stimuli seem to be suppressed is the period of the first quarter. Beginning at full moon the heavy spawning is sometimes completed before new moon but more frequently not until near first quarter. In many respects *Cumingia* resembles *Toxopneustes*, the Beaufort sea urchin, whose periodicity was described by Tennent. The production of gametes in this species is rapid and the gonads are soon replenished after spawning occurs.

It should be noted here that there is a distinction between stimuli which bring about the act of spawning and those which bring about the maturing of the gametes. The latter may be an evenly continuous process or it may be enhanced at certain times. The former is merely the act of extruding these gametes and may be induced by some external stimulus, such as those due to the moon in its various phases, or to any other external stimulus.

In the following paragraphs I give the history of this study of *Cumingia* as constituting an important part of the experimental basis for the conclusions drawn.

During the greater part of the breeding season of *Cumingia*,

no lunar periodicity in spawning is likely to be observed unless attention is directed to it. *Cumingia* has been used as a source of embryological material for class work for many years and only rarely has difficulty been experienced in obtaining eggs in abundance at any time that they have been needed. It appeared that eggs could be obtained at any time during the summer. It was only after receiving a suggestion from Heilbrunn that any convincing evidence of periodic spawning was obtained. He said that *Cumingia* would spawn a second time after its season had apparently ended in August. Morgan gives the spawning season of *Cumingia* as June, July, and August, so that he evidently overlooked the September spawning after the break in August.

It was at Heilbrunn's suggestion that I undertook experiments late in the summer of 1922, when, as he said, a break could be expected.

During the week of August 23 to September 1, 1922, no eggs could be obtained from *Cumingia*, although the characteristic spawning reactions were carried out as usual. The siphons were extended to great length and the whip-like lashing of these organs was carried out. All that was expelled, however, was a considerable amount of mucus containing at best a few immature or defective eggs. There were no mature eggs in the ovaries.

Bean, working in the same laboratory, was likewise unable to obtain eggs during this period for experimental purposes. Bean worked constantly with *Cumingia* during the summer of 1922 and he thought that he detected a periodicity during the height of the breeding season. The maximum spawning periods, according to his statement, occur at new and full moon, being therefore bimonthly. This is interesting, if correct, as indicating that the spawning stimulus might be associated with the tides rather than with moonlight. Further study during the summers of 1923, 1924, 1925 and 1926 shows conclusively that the maximum spawning comes at full moon and is not bimonthly.

The table for 1922 gives the exact data as obtained by the writer. It will be seen that spawning activity is revived somewhat before full moon after a period of complete cessation. Furthermore a careful study of the tables for the years 1922, 1924 and 1926 shows that the heaviest spawning occurs at the

period of the full moon until new moon, and that the period of the first quarter is the period of restricted spawning. This behavior of *Cumingia* can scarcely be explained on any other ground than as a lunar effect.

There is no adequate way of describing the variations in spawning that are so obvious to the experimenter. However, the tables of data appended to this section, together with the comments written at the time the experiments were performed, must suffice to explain the basis of the conclusions arrived at. Although they are long, there seems to be no way to give the evidence more briefly. I give the data for three years only as representative.

Discussion.

There has been much speculation about the cause of lunar periodicity in spawning, but it has for the most part remained speculation and guessing. It can never be solved except by experiments similar to those devised by Mayer. Neither *Cumingia* nor *Chætopleura* is suitable for a study of the causes which have produced lunar periodicity. In the first place, periodicity is not clear-cut in these species and in the second place they can be observed only under laboratory conditions which have been shown to be unreliable. They are affected by shock whereas some species are apparently not so affected.

A casual survey of known cases of lunar periodicity shows a general similarity in all and it is likely that the underlying causes are the same in all. A study which undertakes to explain these phenomena should include a search for other cases and especially all worms that are known to show a lunar periodicity should be studied in detail for purposes of comparison. However, speculation and comparison can scarcely explain it. There is need for experimental methods and the Palolo worm, and the Suez sea urchin or possibly *Nereis limbata* are among the most favorable species for study. It should be a comparatively simple problem to subject them to artificial light, especially polarized light, in addition to all the light to which they are accustomed, to see if they can be thrown out of tune. The Palolo worm seems to offer the best opportunity because of its definite and predictable

time of spawning, so that any change from this time could be regarded as an experimental modification.

Summary.

1. The spawning season of *Cumingia* extends from the middle of June until the middle or end of September. The most active spawning usually includes the last week in June and ends about August 20. Each female spawns more than once and the production of gametes is practically continuous.

2. The spawning is heaviest from full moon to new moon and least at first quarter thus showing a lunar periodicity.

3. *Cumingia* is greatly affected by shock and rough treatment so that sexually mature individuals spawn promptly when brought to the laboratory, after being placed in a bowl of quiet sea water.

4. There are apparently no chemical stimuli by which the sexes stimulate each other to sexual activity as is the case in *Nereis limbata* and the oyster. They spawn as readily when isolated as when in close proximity.

5. It is shown that temperature is not the only factor which determines the duration of the spawning season and periods of spawning.

SPAWNING EXPERIMENTS. 1922.

Cumingia tellinoides.

Table No. I.

Cumingia spawned vigorously during the first half of August but stopped suddenly about August 15. Several investigators were experimenting upon Cumingia eggs at that time and noted this fact. All collections were made at low tide or within an hour of low tide at the convenience of the supply department of the laboratory.

* *September 21, new moon.*

I. 8/23. No eggs spawned. 1 male shed sperm abundantly. 17 did not spawn.

II. 8/24. No eggs and no sperm.

III. 8/27. No eggs spawned. 4 males shed sperm small amount. 14 did not spawn.

* *September 28, first quarter.*

IV. 9/1. 5 females spawned in quantity. 6 males shed sperm, 3 in quantity, 3 in small amount. 7 did not spawn.

Comment.—This revival of spawning after the spawning season had apparently ended is surprising. The quantity of eggs shed is equal to mid-season spawning.

V. 9/2. 4 females spawned in quantity, 2 maximum, 2 one half maximum. 5 males shed sperm, 3 heavy and 2 light. 9 did not spawn.

* *September 5, full moon.*

VI. 9/5. 6 females shed eggs, 4 of them maximum. 5 males shed sperm, 3 abundantly and 2 light. 4 did not spawn.

VII. 9/6. 6 females spawned, 4 maximum, 2 one half maximum. 5 males shed sperm, none maximum, mostly light. 8 did not spawn.

Note.—This cessation or reduction of spawning in August is typical as shown by five years' experience. Spawning is also regularly revived in September. It is most marked when full moon falls during the first week of the month; and less marked when it is near the middle of the month. This record for 1922 led the writer to study these phenomena in detail in succeeding years. They can scarcely be explained on any other ground than a lunar cycle effect.

SPAWNING EXPERIMENTS. 1924.

Cumingia tellinoides.

Table No. II.

* *July 2, new moon.*

I. 7/5. 6 females spawned well (maximum). 5 males shed well (maximum). One did not spawn.

II. 7/8. 4 females spawned well (maximum). 5 males shed well (maximum). 3 did not spawn.

* *July 9, first quarter.*

III. 7/9. 100 per cent. spawned. 3 females spawned, 2 heavy, one light. 4 males shed, 2 heavy, 2 light.

Remark.—Heavy spawning activity during the past week and nearly 90 per cent. of the individuals spawning approximately maximum quantity.

IV. 7/12. 1 female spawned small amount 1/2 maximum. 4 males spawned, 1/2 maximum. 10 did not spawn.

V. 7/14. 6 females spawned, one heavy, 2 medium and 3 light. 9 males spawned, one heavy, 4 medium, 4 light. 10 did not spawn after hours.

Comment.—Light spawning activity compared with ten days ago which was remarkably heavy. Tendency to spawn reluctantly, some individuals spawning only after hours. One spawned after two hours, others slow to act. Only one good lot of eggs from twenty-five individuals, and none on July 12.

* *July 16, full moon.*

VI. 7/19. 4 females shed, 2 maximum, 2 light. 5 males shed, 3 maximum, 2 light. 6 did not spawn.

Comment.—Impression of light or medium spawning activity.

* *July 23, third quarter.*

VII. 7/25. 12 females shed, 5 maximum, 3 medium, 4 light. 7 males shed. Some very heavy, some light. 5 did not spawn.

Comment.—Impression of heavy spawning activity.

VIII. 7/31. 7 females shed, 4 heavy, one medium, 2 very light. 7 males shed, 5 heavy, 2 light. 2 did not spawn.

Comment.—Impression of heavy spawning activity, but not quite as heavy as at last new moon.

* *July 31, new moon.*

IX. 8/2. 5 females shed, 3 heavy, 2 light. 6 males shed, 4 heavy, 2 light. 7 did not spawn.

Comment.—Only one female spawned within an hour. And only two males within the first hour. (Why?) No others spawned for two and one half hours. Impression of poor spawning activity, reluctant and evident retention of eggs from some cause, also low percentage spawned after hours.

* *August 7, first quarter.*

X. 8/12. 11 females shed, 5 near maximum, 2 medium, 4 light. 14 males shed, all heavy or medium. 2 did not spawn within the first hour.

Comment.—This lot began to spawn after fifteen minutes, males more quickly than females. Most of the males began before thirty minutes. Some females began after twenty-five minutes but most of them after 40 minutes. Fourteen males and eight females had shed within one hour. Three females spawned after one and one half hours.

Impression of good heavy spawning activity and the reluctance observed a week ago was gone. The spawning was unusually prompt and vigorous. Cause?

* *August 14, full moon.*

XI. 8/16. 100 per cent. spawned. 6 females shed, 4 heavy, 2 medium. 3 males shed, 2 heavy, one medium. 1 spawned after 1½ hours heavily.

Comment.—Very heavy spawning activity. All except one spawned within an hour. Most of them began in 20 to 30 minutes. The last one spawned heavily after one and one half hours.

Heilbrun collected twelve Cumingia this date. All but one spawned.

XII. 8/20. 6 females spawned within an hour, 4 heavy, 2 medium. 13 males shed, 7 heavy, 3 medium, 3 light. 2 females spawned after 1½ hours. Very few eggs from these two. 1 did not spawn.

* *August 22, third quarter.*

XIII. 8/26. 100 per cent. spawned. 12 females shed during first hour, 5 heavy, 2 medium and 5 a mere trace. 9 males shed, 5 heavy, four light. 2 females spawned after one hour and forty minutes. The last two spawned very few eggs.

Comment.—Impression of heavy spawning activity during the past ten days. Five females gave only a very few eggs which indicates recent spawning on their part at the full moon period.

The inhibition to spawning noticed earlier is not now working. The heaviest spawning of the year has occurred during the past ten days.

XIV. 8/29. None spawned during the first hour. 4 females spawned a few eggs later, 1/10 maximum. 2 males shed a trace of sperm. 14 did not sperm.

XV. 8/30. 2 females spawned. Very few eggs, approximately 1/15 maximum. 3 males shed lightly, 1/10 maximum. 15 did not spawn.

* *September 1, new moon.*

XVI. 9/2. 5 females shed a few eggs, scarcely visible in the dish (a trace). 2 males made water slightly turbid with sperm. 18 did not spawn.

Comment.—Spawning negligible. Experiments for 1924 ended at this time. Experience has shown that spawning no doubt revived at the approach of the September full moon for a few days. This series of experiments shows that spawning was heavy early in July from the time the experiments of the season

began until after the first quarter when they suddenly fell off not to be fully revived until somewhat after full moon. Then followed a week of heavy spawning until new moon.

Shortly after the first quarter in August the heaviest spawning of the year began and continued until the approach of the next new moon or from August 7 to August 29. Then came the usual late season, temporary cessation of spawning which presumably revived somewhat at the September full moon.

It is unfortunate that these experiments could not have begun by the middle of June and continued until the middle of September. The experience of several years leads one to believe that spawning began at full moon in June and reached maximum late in June. The variations in spawning activity under laboratory conditions show that the production of the gametes is greatest around full moon and least around the first quarter. These results are shown regularly except at the height of the breeding season when it may be masked by the rapidity of egg production.

SPAWNING EXPERIMENTS. 1926.

CUMINGIA TELLINOIDES.

Table No. III.

The following table of experiments shows the duration of the spawning season and variations in spawning activity during the lunar cycle. From fifteen to twenty-five individuals were used in each experiment. They were isolated in stender dishes containing enough water to cover them fully. Spawning usually occurs within an hour. Collections were always made at low tide.

I. 3/1. No eggs and no spermatozoa.

II. 4/1. No eggs and no sperm.

III. 6/1. No eggs and no sperm. (Experiment by Hugh Montgomery.)

IV. 6/11. No eggs and no sperm. (Experiment by Hugh Montgomery.)

V. 6/15. No eggs, one male shed active spermatozoa, one fifth maximum.

* *June 18, first quarter.*

VI. 6/21. 2 females spawned, approximately 1/5 maximum. 2 males shed sperm, 1/4 maximum. 8 did not spawn.

Comment.—Eggs mature, cleavage normal. Spawning season opened between June 15 and June 20.

* *June 25, full moon.*

VII. 7/1. 6 females spawned, 1/4 to 1/3 maximum. 7 males shed sperm, 1/3 to 1/2 maximum. 2 did not spawn.

* *July 2, third quarter.*

VIII. 7/6. 9 females spawned rather abundantly, 1/2 to 3/4 maximum. 12 males shed sperm, 11 heavy, 1 light. 2 did not spawn.

* *July 9, new moon.*

IX. 7/15. 2 females spawned, 1/2 to 2/3 maximum. 12 males shed sperm, 10 medium, 2 light. 12 did not spawn.

Comment.—Light spawning, and many failed to spawn.

* *July 17, first quarter.*

X. 7/19. 2 females spawned 1/20 maximum (very few eggs). 4 males shed sperm, very light, 1/10 to 1/20 maximum, scarcely detectable in the dish. 13 did not spawn.

Comment.—Spawning all but extinct. Not even one fair lot of eggs obtained. Scarcely visible in the dish. Almost no spawning activity during the past week.

XI. 7/24. 13 females spawned lightly, 1/2 to 1/3 maximum. 7 males shed sperm, 6 heavy, 1 light. 2 did not spawn.

Comment.—Great revival of spawning but light in amount.

* *July 25, full moon.*

XII. 7/26. 8 females spawned, 4 heavy (maximum) and 4 medium (1/2 to 2/3 maximum). 12 males shed sperm, 6 heavy, 4 medium, and 2 light. 6 did not spawn.

Comment.—Very heavy spawning. The heaviest this year to date. All spawned promptly after thirty or forty minutes. Lunar periodicity is demonstrated clearly. Almost complete cessation of spawning ten days before full moon but came on again actively at full moon.

* *July 31, third quarter.*

XIII. 8/4. All spawned actively, 100 per cent.

* *August 8, new moon.*

XIV. 8/10. 6 females spawned, 3 heavy, 2 medium, 1 light. 11 males shed sperm, 10 heavy or medium, 1 light. 6 did not spawn.

XV. 8/13. 7 females spawned, 6 medium or heavy, 1 light. 7 males shed sperm, mostly heavy. 1 did not spawn.

XVI. 8/16. 100 per cent. spawned. 8 females spawned in quantity. 7 males shed sperm in quantity.

* *August 16, first quarter.*

XVII. 8/20. 5 females spawned (1/2 maximum or less). 7 males shed sperm, 1/2 maximum. 2 did not spawn.

XVIII. 8/21. 6 females spawned heavily, 1/2 to 3/4 maximum. 9 males shed sperm heavily. 5 did not spawn.

* *August 23, full moon.*

Comment.—There is no evidence of reduced spawning at the first quarter in August, whereas in July there was a clear-cut case.

XIX. 8/28. 7 females spawned, approximately 1/2 maximum. 12 males shed sperm, medium to light. 5 did not spawn.

Comment.—Spawning at this time by about 80 per cent. of the individuals but for the most part below the spawning of last week in quantity. Good lots of eggs still obtained, 1/2 to 1/3 maximum the rule. The quantity of eggs spawned by each individual is usually reduced toward the end of the season.

* *August 29, third quarter.*

XX. 8/31. 3 females spawned, 2 1/10 maximum, 1 1/20 maximum. 4 males shed sperm (1/4 maximum). 25 did not spawn.

XXI. 9/1. 6 females spawned 1/20 maximum (scarcely visible in the dish). 4 males shed sperm, very light, 1/10 maximum, water only slightly turbid. 14 did not spawn.

XXII. 9/3. No eggs and no sperm. Some extended the siphons and lashed them in characteristic manner but there were no eggs in the ducts.

Comment.—Very light spawning. Almost none since August 28. Spawning suddenly fell off, one week after full moon. Vigorous spawning continued for five days after full moon.

* *September 7, first quarter.*

XXIII. 9/9. 6 females spawned, 1 2/3 maximum, 4 1/5 maximum, 1 1/10 maximum. 5 males shed sperm, 3 1/2 maximum, 2 light. 19 did not spawn.

Comment.—Considerable revival of spawning noted. Much more active than last week, but still relatively insignificant. Good for so late in the season.

* *September 21, full moon.*

XXIV. 9/21. 3 females spawned, 1 maximum, 1 1/5 maximum, 1 1/10 maximum. 2 males shed sperm, 1 3/4 maximum, 1 1/2 maximum. 15 did not spawn.

Comment.—Revival of spawning evident, though not extensive. Quantities surprisingly large. Temperature 18° C., or 17½° C.

XXV. 9/10. Temperature 17° C. No eggs and no sperm.

Comment.—The spawning season of Cumingia ends between September 15 and October 1. This year spawning continued until the end of September. The foregoing data give no very definite information about the time of spawning in nature. It is evident that eggs are produced almost continuously although probably not uniformly. There is a lunar periodicity either in the production of the gametes or in the spawning or in both. Spawning by each individual occurs more than once during the season.

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CYTOLOGY OF SACCHAROMYCES CERVICIÆ WITH ESPECIAL REFERENCE TO NUCLEAR DIVISION.

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For some years following the discovery of cell division it was thought that direct division was responsible for the duplication of the nucleus, and even after the discovery of indirect division the former was still considered to be the usual method and mitosis was held to be a peculiar and infrequent phenomenon. Some held that the two processes were related and that mitosis was derived from the simpler and more primitive one, amitosis. However, as the investigation of this problem was continued evidence was gradually accumulated to show that mitosis is the usual if not universal method of cell division in higher animals and plants, and that amitosis is not a reproductive phenomenon at all, but has for its function the increase of nuclear surface in relation to volume and is rarely, if ever, followed by cytoplasmic division. This conception was well expressed by Conklin (1917) in the following passage, "Mitosis and amitosis are fundamentally unlike. Mitosis is the one and only method of bringing about equal division and distribution of the chromatic material of the nucleus. Amitosis is not a genuine divisional phenomenon at all, but merely a means of increasing the nuclear surface and of distributing nuclear material throughout the cell, comparable to nucleur lobulation, fragmentation or distribution. These two processes are not equivalent or even comparable, nor may one of them be converted into the other." Although this view is not universally held it is very generally accepted by cytologists at the present time.

The study of cell reproduction is much more difficult in Protozoa than in higher forms, as many of the types of intranuclear division have a superficial resemblance to amitosis. Minchin (1912) actually accredits the description of direct division in a number of protozoa, although he admits that some

of the formerly supposed cases of amitosis have been shown to be merely simulations of it. Some of the authorities in this field, however, are willing to elevate the Protozoa to essentially the same position as Metozoa in this respect. Kofoid (1923) says: "In the first place amitosis as described in the Protozoa is either a pathological or degenerative process, as it is in the Metozoa, or it is based on a partial account of the normal process of mitosis in which the nuclear membrane remains intact throughout the whole process, as it does in the flagellates and rhizopods, and in its anaphases presents a superficial resemblance to pathological amitosis. The persistence of the nuclear membrane in no way interferes with the occurrence of chromosomes constant in number and kind. In other words the doctrine of chromosome continuity, in so far as amitosis is concerned, is no more affected in the Protozoa than in the Metazoa." The nuclear division (promitosis) of many of the lower protozoa is unquestionably very different from the mitotic division exhibited by higher plants and animals, but it seems likely that in all cases it is a simplified form of mitosis and is entirely unrelated to amitosis, even though it does superficially resemble it.

It seems as though the difficulty of studying nuclear division in any form is responsible for the persistence of investigators in describing it as amitotic. The tapeworm *Monezia* offers an example of this (Child, 1911). Yeast belongs in the same category, and we should realize the extreme difficulty of investigating this problem in yeasts by considering the minute size of the cells and the fact that for some time there was a heated controversy as to whether or not they even possess a nucleus. Some of those who took the affirmative, as has since been proved, were describing structures which belong to the cytoplasm. Wager (1898) and Wager and Peniston (1910) described as the nucleus, the actual nucleus, the vacuole, and a part of the metachromatic material surrounding the nucleus and vacuole. The division of this compound structure was described as amitotic in the case of budding and by an "intermediate step in karyokinesis" in the case of spore formation. This account of the indirect division of a cytoplasmic vacuole is comparable to the early figures of mitosis of the parabasal body (kineto-

nucleus) in trypanosomes and shows that without a favorable modification of technique the problem is almost invincible.

Some of the early contributors to our knowledge of the cytology of yeasts unquestionably saw and illustrated, with a fair degree of accuracy, not only the resting nucleus but also stages in its division. Janssens (1902) and Janssens and Leblanc (1898) considered the division of the nucleus to be an intermediate form of mitosis. Swellengrebel (1905) and Fuhrmann (1906) as a true mitotic process. Although these last two articles are in the main correct they have not been generally approved, and the ideas of Guilliermond (1904, '12, '17 and '19), which gain weight by the mere bulk of his work on yeast, seem to meet with more favor. This author, who is responsible for a large part of our knowledge of the well developed sexuality of yeasts and for an excellent account of the typical metazoan mitosis found in spore formation in *Schizosaccharomyces octosporus*, maintains that in bud formation the nucleus divides by a process identical with amitosis in the tissue cells of higher organisms, where, as Conklin asserts, it is not a reproductive phenomenon at all. To accept this would be to admit that mitosis and amitosis are fundamentally alike and interchangeable. This would undermine a large part of our knowledge of cytology and genetics. The problem most assuredly warrants critical study.

Methods.

Pure cultures of *Saccharomyces cerviciæ* were used for this work. They were cultivated on both liquid and solid media. French proof broth was used for the liquid medium, French proof agar for the solid.

The organisms were transferred to slides which had been previously smeared with albumen fixative and the moist films were fixed either in corrosive-acetic-alcohol (95 per cent. alcohol saturated with mercuric chloride 95 parts, glacial acetic acid 5 parts) or Bouin's solution (saturated picric acid solution 75 parts, formalin 20 parts, glacial acetic acid 5 parts). Iron-alum-hæmatoxylin counterstained with light green or not counterstained at all was found to be the best means of staining. Delafield's hæmatoxylin and carbol fuchsin were tried without

success, and eosin and orange G were found to be equally useless.

Fixation in corrosive-acetic-alcohol slightly shrinks the cells and gives the chromatic material in the cytoplasm such great affinity for basic stains that in most cases the nucleus is obscured. After fixation in Bouin's it does not stain so heavily and the nucleus can be seen in all cases, providing it is not too heavily stained. The successful technique finally developed was fixation in Bouin's and staining before the picric acid was completely washed out. This decreased the affinity of both nuclear and cytoplasmic chromatic material for stain, especially the latter. In this way the nucleus could be stained without showing the chromatic bodies in the cytoplasm at all. This procedure made possible a careful study of the nuclear chromatin both at rest and during division. Light green was found very valuable for the study of the metachromatic granules. Even when they are very abundant this stain will demonstrate their granular nature. In preparations in which no counterstain or the other acid stains are used they frequently appear as a single, large mass.

It was found that fixation and staining in carbol fuchsin followed by light green furnished an excellent method for demonstrating the alveolar nature of the cytoplasm. Although the nucleus could be seen in these cells its minute structure was not visible and the method is of no value for the present work. Light green is taken up by the gelatinous secretion sometimes given off by the yeast cells and consequently furnished a good means of demonstrating the presence of this substance.

Morphology.

Saccharomyces cerviciæ is a round to slightly oval yeast whose size ordinarily ranges from 5 to 10 micra. In old cultures the cells are occasionally elongated, sometimes crescentic (Fig. 7). The cell is enclosed in a thin though distinctly evident wall. This wall occasionally becomes greatly thickened and the cells pass into a very resistant stage (durable cells, chlamydospore) such cells can be kept dry for a year without destroying their viability (Figs. 10 to 13).

One of the most prominent structures inside the cell is the

vacuole, small or entirely absent in very young cultures, but attaining a relatively great size after several days. In some cases it almost entirely fills the cell (Fig. 2). This vacuole is generally round, with a very regular outline. The cytoplasm is distinctly alveolar and contains numerous metachromatic granules. Occasionally cells are found with none of these granules, especially in very young cultures (Fig. 5) and sometimes there are only a few present, in which case they are located near the nucleus (Fig. 4). In old cells with a large vacuole there is frequently such a great mass of metachromatic material surrounding the nucleus that the latter body is obscured (Figs. 2 and 3), and the granular nature of the metachromatin is not discernible. Lines of fine granules can be seen leading from this mass to large granules in other parts of the cell, thus converting the metachromatic material into a connected unified system. These granular strands are especially evident where they go around the vacuole, and constitute the nuclear reticulum of Wager. Guilliermond figures basophilic granules within the vacuole. It is these granules above and below the vacuole which he sees and there are really no granules within the large vacuole. When material is fixed in Bouin's solution and stained in iron-alum-hæmatoxylin without washing out the picric acid the metachromatic granules do not stain. When this procedure is properly carried out, the well stained nucleus embedded in the alveolar cytoplasm is quite evident and easily studied. (Figs. 6 to 8.)

The nucleus of *Saccharomyces* is quite similar to that of higher plants, particularly *Phaseolus* (Kater, 1926). The nuclear membrane is equally as evident as in higher plants. Centrally located is a large basophilic nulceolus or karyosome from which radiate slender slightly basophilic linin strands. These strands run from the nucleolus to the nuclear membrane in identically the same manner as in *Phaseolus*. There are generally about six such strands visible. Just inside the nuclear membrane are located a number of chromatin granules. The larger ones are found at the points where the linin strands come in contact with the nuclear membrane. Except for the minute size of the nucleus (1 to 3 micra in diameter) it is hardly distinguishable

from that of *Phaseolus*. A fruitful study of the finer points of the structure of the resting nucleus as well as following it through division would have been impossible without some means of staining it without affecting the metachromatin. It is this feature of our technique which makes the observations convincing. A centrosome could not be identified in the interkinetic cell nor could any variation in the cytoplasm surrounding the nucleus, comparable to sphere substance, be seen.

The yeast cell contains both fat and glycogen, the former usually in small globules in the vicinity of the nucleus.

It has been previously observed that yeast cells will give off a gelatinous secretion when permitted to dry gradually in a closed vessel. This is thought to play a part in the agglutination of yeast and the consequent clearing of the medium. In the present work it was found that dilution of the medium of an old culture with distilled water will produce this secretion (Fig. 9). The secretion has great affinity for light green.

Budding.

In young cultures the usual method of reproduction is by budding. The superficial features of this process are matters of common knowledge, and consequently, this description will be limited to the internal phenomena. The bulge in the cell wall and the entrance of cytoplasmic elements into the bud occurs some time before any change in the resting nucleus is observable (Fig. 14). The finely vacuolated cytoplasm is the first material to enter the bud. This is followed by the metachromatic granules. In those cells that contain a large vacuole the bud is generally formed near the nucleus and consequently near the greater part of the metachromatic material. The mass of this material near the bud separates into individually visible granules, part of which migrate through the isthmus into the bud, the rest moving to the opposite side of the parent cell (Figs. 15 and 21). Whether or not they divide at this time cannot be stated. Even after these granules are distributed to the two cells the nucleus is still in the resting condition. The large clear vacuole does not divide, but after the bud has attained almost the size of the parent cell a small vacuole appears within it and gradually enlarges.

After the bud has attained almost normal size and the distribution of metachromatic granules is completed the nucleus gives evidence of impending division. The chromatin accumulates on the linin strands, making them very evident and extremely basophilic. At the same time the disperse chromatin granules enlarge and the whole nucleus becomes a perfect miniature of an early prophase in *Phaseolus vulgaris* (Kater, 1926, Figs. 15 and 16). Meanwhile the nucleus remains stationary (Fig. 16). The steps in the transformation of such a nucleus into chromosomes cannot be followed with any degree of certainty, but many stages such as the one represented in Fig. 17 were seen. A knot of chromosomes probably occupying the old location of the nucleolus is here observed at the periphery of which individual chromosomes are becoming disentangled from the rest.

So far neither a dividing centrosome or spindle can be identified. However, as the chromosomes become arranged on the metaphase plate a spindle becomes visible (Fig. 18) and in some cases the ends are lodged in a granule perfectly comparable to the centriole in metazoa. No astral radiations are visible (Fig. 19). Such a centrosome has previously been reported in yeast (Swellengrebel, 1905; Guilliermond, 1917). In Fig. 18, the chromosomes are quite clearly separating in the same way as they do in higher plants and animals. Except for the difference in size the early anaphase represented in Fig. 19 could easily be mistaken for a metazoan mitotic figure. No careful attempt was made to count the chromosomes of *Saccharomyces* as their size makes it impractical. However, it seems certain that there are more than the four reported by Swellengrebel (1905) and Fuhrmann (1906). Probably at least twice that number.

The migration of the chromosomes through the isthmus into the bud is not at all clear. In some figures the orderly arrangement of the anaphase plates seems to be maintained (Fig. 22) while in others the chromosomes appear to enter the bud one at a time. In such cells the spindle disappears and only the irregularly arranged chromosomes can be seen (Fig. 21). In overstained slides specimens similar to the one illustrated in Fig. 22 very greatly resemble amitosis, and are probably responsible for the confusion on this point.

After being distributed to the two daughter cells the chromosomes become collected at one point and apparently fuse forming a uniformly basophilic mass in which the individual chromosomes cannot always be identified. The imbibition of achromatic material results in the alveolization of the outer part of this mass. The limiting membrane (nuclear membrane) is frequently bulged between the linin strands (Fig. 25). A continuation of alveolization results in a spherical nucleus with a nucleolus of moderate size, the resting condition.

The division of the cytoplasm in the isthmus occurs shortly after the separation of the chromosomes and the disappearance of the spindle (Fig. 24). Although the cytoplasm has separated, the cell wall frequently does not divide for a considerable length of time and holds the two cells together. In this way a number of cells are sometimes connected, somewhat resembling a mycelium (Fig. 8).

No attempt has been made to study the formation of ascospores, but according to previous accounts the nucleus divides by mitosis.

Discussion.

The above account leaves two points without adequate solution, namely the transformation of the nucleus into chromosomes and the migration of chromosomes through the isthmus. Fortunately these are matters of detail and the remainder of the account shows conclusively that the nucleus of yeast, in the formation of buds, does not divide by constriction, but that chromosomes are formed, divide (Fig. 18), separate, and give rise to daughter nuclei in the same way as in higher organisms, and yeast can be placed in the same category with higher animals and plants, and protozoa as enunciated by Conklin and Kofoid respectively.

To accept Guilliermond's descriptions of the indirect division of the nucleus in the formation of ascospores and direct division in budding would be to admit the reproductive nature of amitosis and, secondly, that a nucleus produced by this method may later divide, in the ascus, by perfectly normal mitosis. This would make untenable many of the generalized conceptions of

the cytologist and the geneticist. It seems remarkable that the investigation of nuclear division in yeast has not previously attracted the attention of cytologists, as well as mycologists.

In the earlier work on *Phaseolus* it was found that the linin strands radiating from the nucleolus are actually the linin sheaths of chromosomes which persist through the resting condition and give the chromosomal vesicles morphological individuality during interkinesis. It seems probable that the linin strands of *Saccharomyces* are of the same nature. The collection of chromatin along them in the early prophase (Fig. 16) and the bulge between them in the telophase (Fig. 25) would indicate this. However, since the actual transformation of the prophase nucleus into chromosomes and the alveolization of early telophase chromosomes could not be followed with any degree of certainty a definite statement of chromosomal continuity in yeast cannot be made on a morphological basis as in *Phaseolus*, but the probable homology is certainly worthy of mention.

The existence of radiating linin strands alone would not justify this interpretation, since they are present in many protozoan nuclei where the nuclear membrane remains intact throughout mitosis. In such organisms the membrane is not a product of the linin sheaths of chromosomes, as in higher animals and plants, and the linin strands could not easily be interpreted in the same manner. Such a nucleus is found in Polytomella citri (Kater, 1925). In *Saccharomyces* the nuclear membrane disappears and consequently it is quite possible that the linin strands are homologous with those of *Phaseolus*.

Summary.

The nucleus of *Saccharomyces cerviciæ* divides by mitosis in the process of budding.

The chromosomes apparently form, divide, separate, and give rise to daughter nuclei in much the same way as in *Phaseolus*. The linin strands connecting the nucleolus and nuclear membrane probably represent sheaths of chromosomal vesicles.

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EXPLANATION OF PLATES.

Figures 1 to 5 and 15, 20, and 21 made from material fixed in corrosive-acetic-alcohol and stained with iron-alum-hæmatoxylin, Figs. 4, 5, and 15 counterstained with light green. The remaining figures from material fixed in Bouin's solution and stained as above. All drawings made with Abbe model camera lucida. Magnification 3,200 ×.

PLATE I.

FIG. 1. Small yeast cell showing fine granular lines of chromatic material in the cytoplasm, nucleus partly obscured.

FIG. 2. Nucleus entirely obscured. Granular lines above vacuole clear.

FIG. 3. End view of a cell similar to Fig. 2.

FIG. 4. Cell containing only a few metachromatic granules. Nucleus visible. Medium-sized vacuole.

FIG. 5. No metachromatic granules. Nucleus very large and structure clear.

FIG. 6. Nucleus above vacuole which is not so clear as in side view.

FIG. 7. Cell exhibiting crescentic form.

FIG. 8. Four connected cells showing manner of connection by unbroken wall.

FIG. 9. Cross hatching indicates gelatinous secretion which holds the cells together.

FIG. 10. Resistant cell. Note heavy wall and abundance of metachromatic granules.

FIG. 11. The same. Metachromatic granules disappeared.

FIG. 12. Resistant cell showing departure from spherical form and shrinking of protoplast.

FIG. 13. Resistant cell that has been kept dry for one year.

Drawings by G. T. Kline.

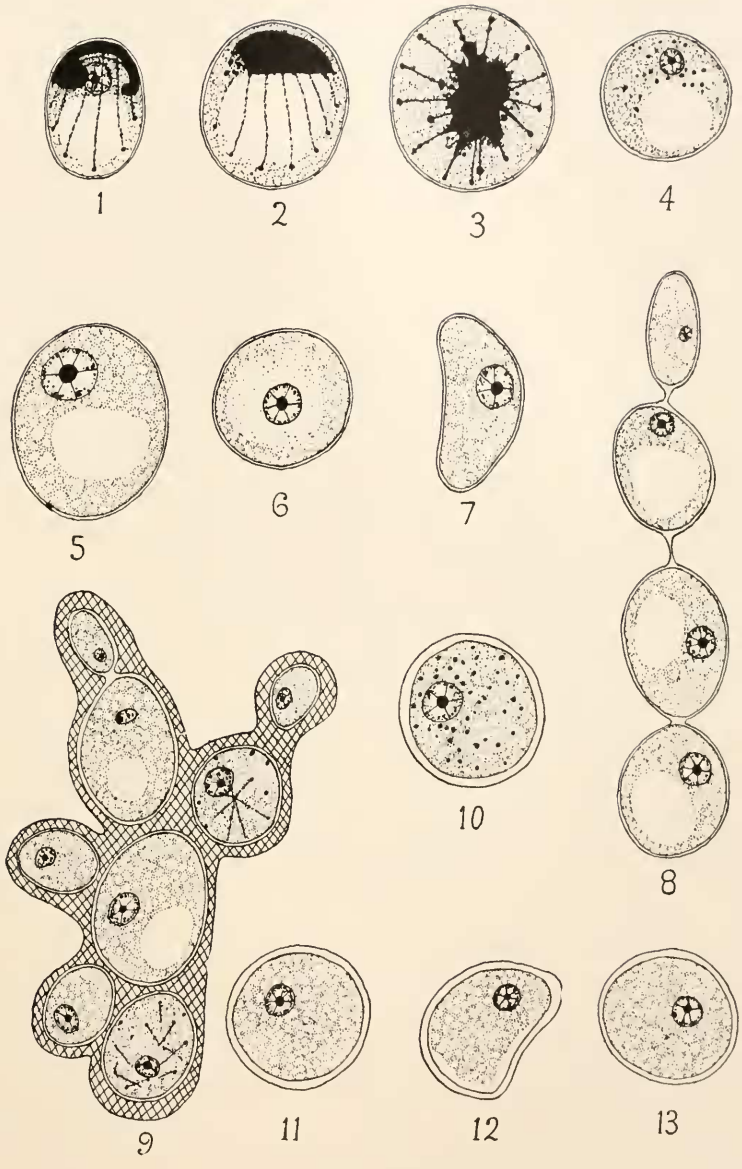

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Plate II.

Fig. 14. Early bud. Extremely large vacuole.

Fig. 15. Metachromatic granules entering the bud. Nucleus not yet beginning division.

Fig. 16. Early prophase. The chromatin is collecting on the linin strands. A few metachromatic granules visible in both cells.

Fig. 17. The nucleus is breaking up into chromosomes. Slightly oblique view making bud appear abnormally small.

Fig. 18. Metaphase. The chromosomes are separating on the equator of the spindle.

Fig. 19. Anaphase. Centrosome and spindle very evident. Metachromatic granules in both cells.

Fig. 20. The same. Slightly later.

Fig. 21. The chromosomes are passing through the isthmus. A few metachromatic granules in opposite ends of both bud and parent cell. These are much smaller than the chromosomes.

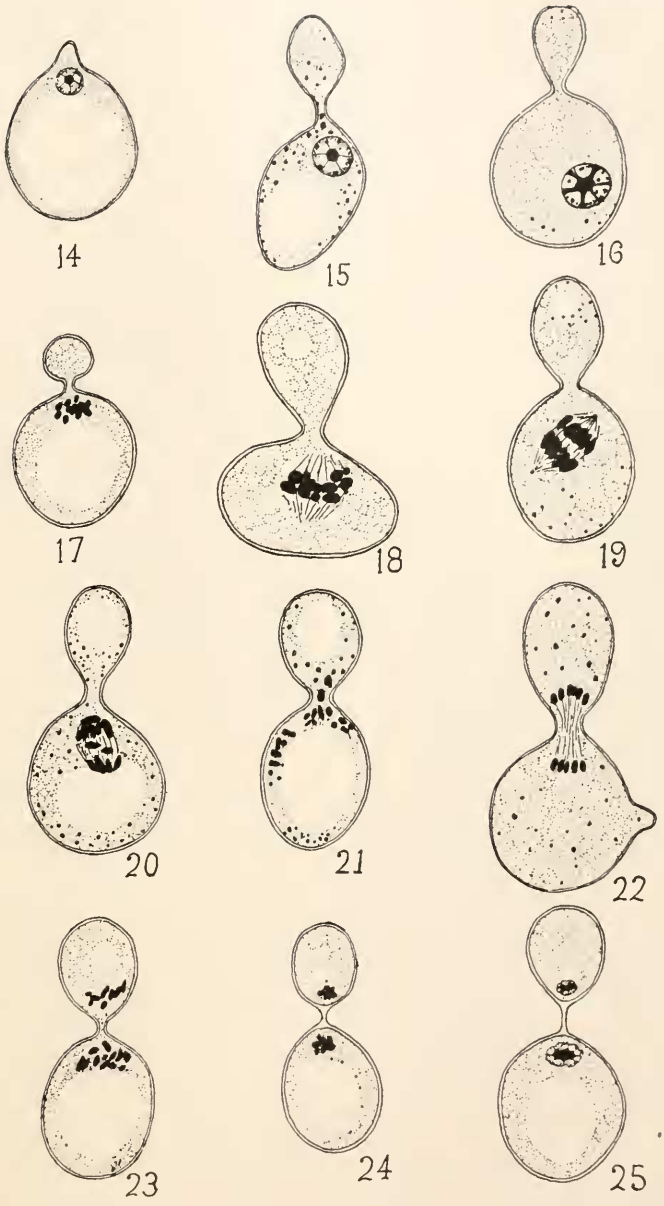
Fig. 22. Late anaphase. Spindle very evident. A second bud on the parent cell.

Fig. 23. The spindle not visible. The isthmus is closing. Chromosomes collected near one point.

Fig. 24. The cytoplasm has divided, the cells being held together by their walls. Chromosomes beginning the apparent fusion.

Fig. 25. Late telophase. Large nucleolus. Note bulges in nuclear membrane between linin strands.

Drawings by G. T. Kline.



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MEASURES OF INSECT COLD HARDINESS.

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Different measures of insect cold hardiness used by different workers may usually be reduced to the empirical survival test. Bachmetjew (1901) used the "vital temperature maximum" or the second time an insect reached the undercooling point. Duval and Portier (1922) considered that there was a freezing point below that ordinarily determined, the higher freezing point being that of the body fluids, the lower that of the body cells.

In strong contrast to the scarcity of measurements of insect cold hardiness, stand the many determinations by plant physiologists. Osmotic pressure as determined by freezing point lowering has been widely used from the time of Sachs and Pfeffer. Water content has been of value as a criterion of cold hardiness in plant groups far separated taxonomically. For example, Johnson (1923) used water content of peach buds as a measure of cold hardiness, and Steinbauer (1926) employed it for clover seeds. Newton and Gortner (1922) and Newton (1924) emphasize the importance of bound water to cold hardiness. Müller-Thurgau (1886) proved conclusively that some plants could survive freezing. The ability of a plant to survive freezing was defined by Harvey (1918) as cold hardiness.

The two kinds of insect cold hardiness (1) hardiness to the quantity factor of low temperature or ability to withstand long periods of relatively mild low temperature and (2) hardiness to the intensity factor of low temperature, or ability to withstand extremes of low temperature have been discussed in a previous paper. In the present paper cold hardiness to the intensity factor alone will be considered.

Closely associated with changes in cold hardiness are changes in moisture content. Insects dehydrated but not to the period of injury, can withstand temperatures far lower than undehydrated individuals. This is strikingly true for insects that are

not self dehydrating to any large extent. Thus the Japanese beetle, *Popillia japonica* Newm., does not exhibit any marked body weight changes over winter when kept in moist surroundings, but can be experimentally dehydrated to half its body weight. When thus treated they are very cold resistant, having a survival temperature of as low as − 28° C. In contrast

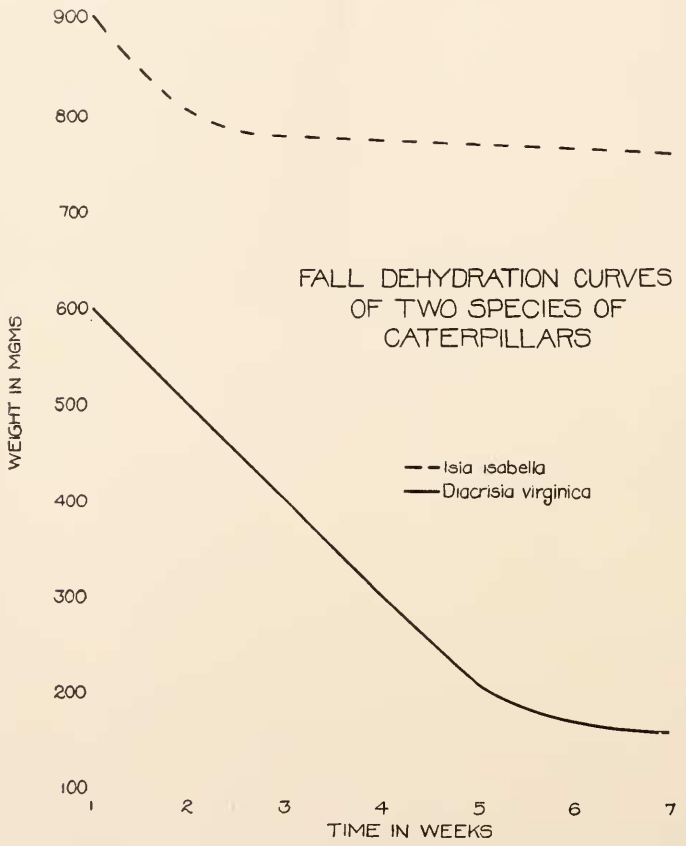

FIG. 1. Fall dehydration curves of two species of caterpillars, *Isia isabella* and *Diacrisia virginica*.

to the Japanese beetle larvæ there are some species of oak borers and caterpillars which are normally self dehydrating during the winter. The dehydration curves (Fig. 1) of *Isia isabella* Hy. Edw. and *Diacrisia virginica* Fabr. show a marked water loss as these caterpillars go into hibernation. At the period of inflection

of the weight loss curve (Fig. 1) these insects can survive freezing. When the curve is plotted with rate against weight loss the point of inflection is brought out more clearly (Fig. 2). Up to

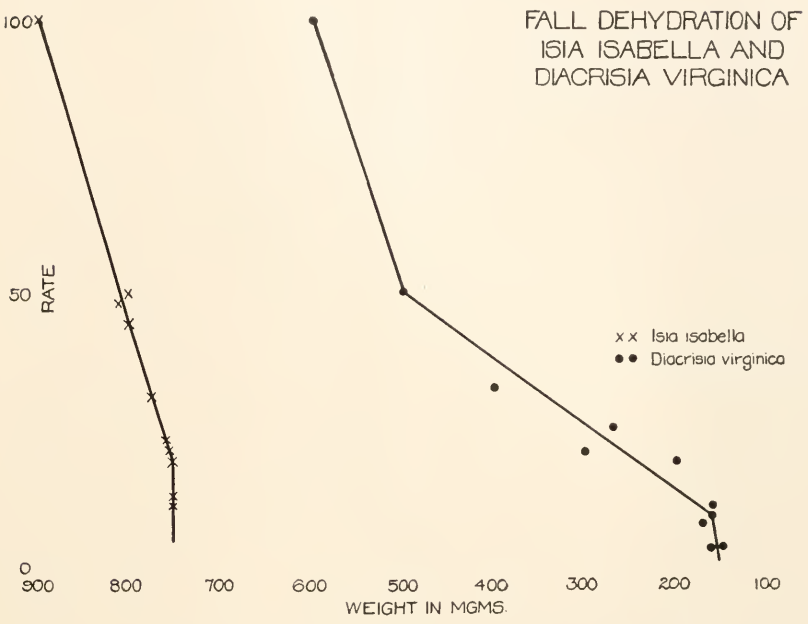

FIG. 2. Fall dehydration of *Isia isabella* and *Diacrisia virginica*. $\frac{1}{\text{time}}$ or rate plotted against weight.

the point of inflection of the weight loss curve the undercooling point of the blood is the minimum survival temperature. Beyond that point the undercooling point no longer measures the total cold hardiness which reaches to below − 40° C. There is no free body fluid on which a conductivity reading can be made.

The oak-borers, *Synchroa punctata* Neum., *Dendroides canadensis* Lec., *Romaleum rufulum* Hald. also are normally self dehydrating but never to the extent of losing all their free water. Although very cold resistant, having survival temperatures of below − 40° C., at no time even in the deepest winter, is it impossible to obtain blood samples. But conductivity is found to be proportional to the survival temperature (Fig. 3). The water content of these insects, obtained by heating them in an oven for four hours at + 50° C., is only relative but does appear

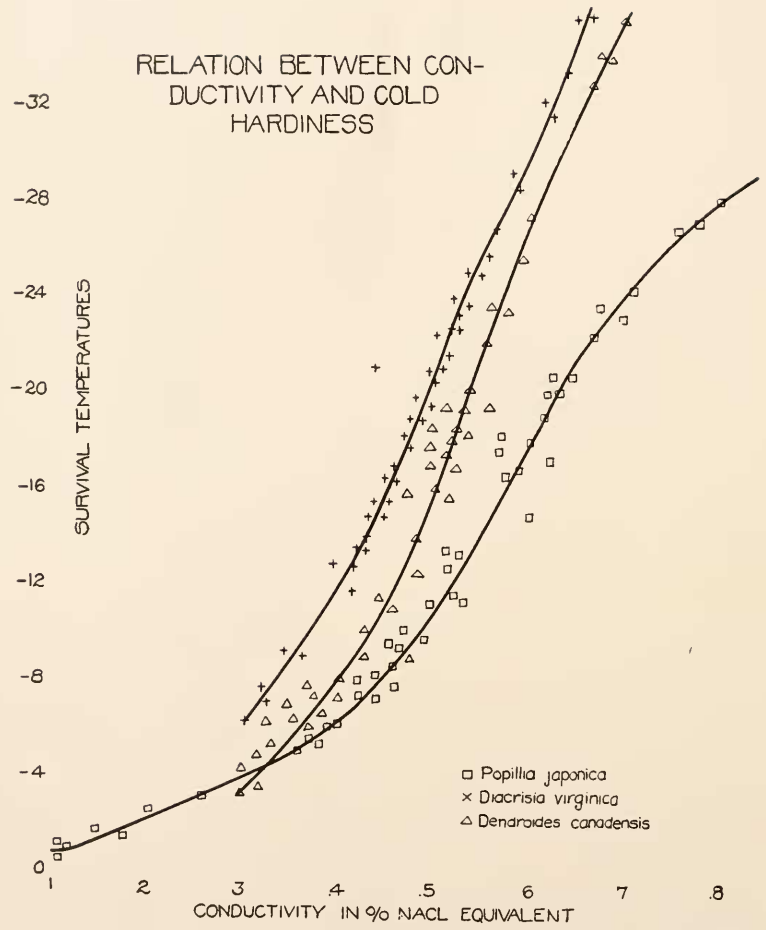

FIG. 3. Relation between conductivity and cold hardiness. *Popillia japonica* □, *Diacrisia virginica* ×, *Dendroides canadensis* △.

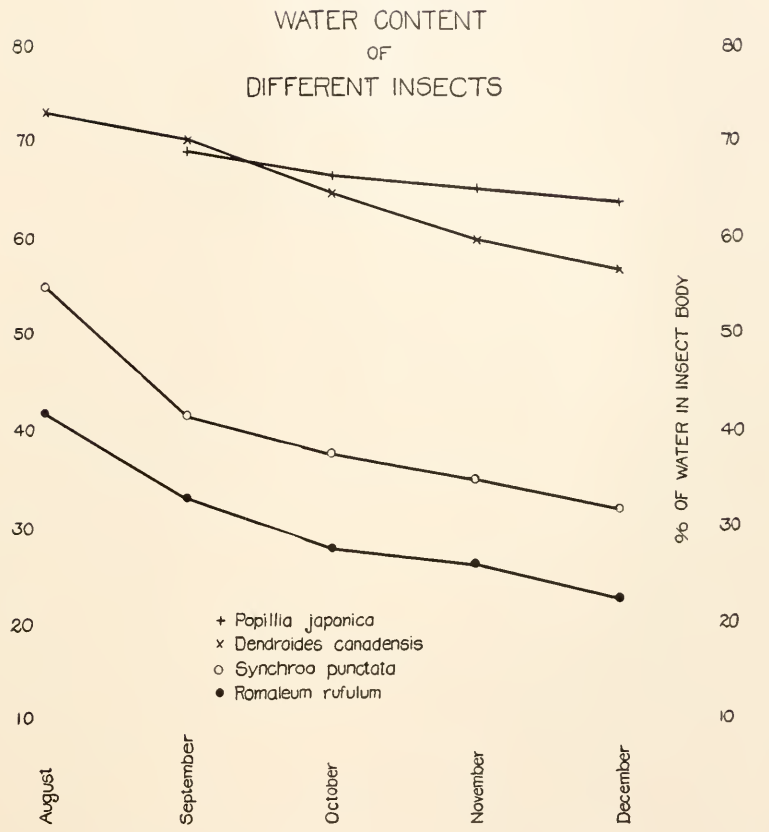

FIG. 4. Water content of different insects during the season at which they develop cold hardiness. *Popillia japonica* +, *Dendroides canadensis* ×, *Synchroa punctata* o, *Romaleum rufulum* ●.

to give comparable results with different species. The per cent. of water before and during hibernation of three species of oak-borers and of the Japanese beetle are shown in Fig. 4.

The Japanese beetle larvæ, *Popillia japonica* Neum. represent an ecological group far more protected than either the oak-borers or the woolly bear caterpillars. This species hibernates in the ground below the frost line. About 97 per cent. are third instar larvæ and about 3 per cent. second instar. There is a cyclic change in the cold hardiness of these larvæ, not as marked, however, as in the oak-borers but more apparent than in the

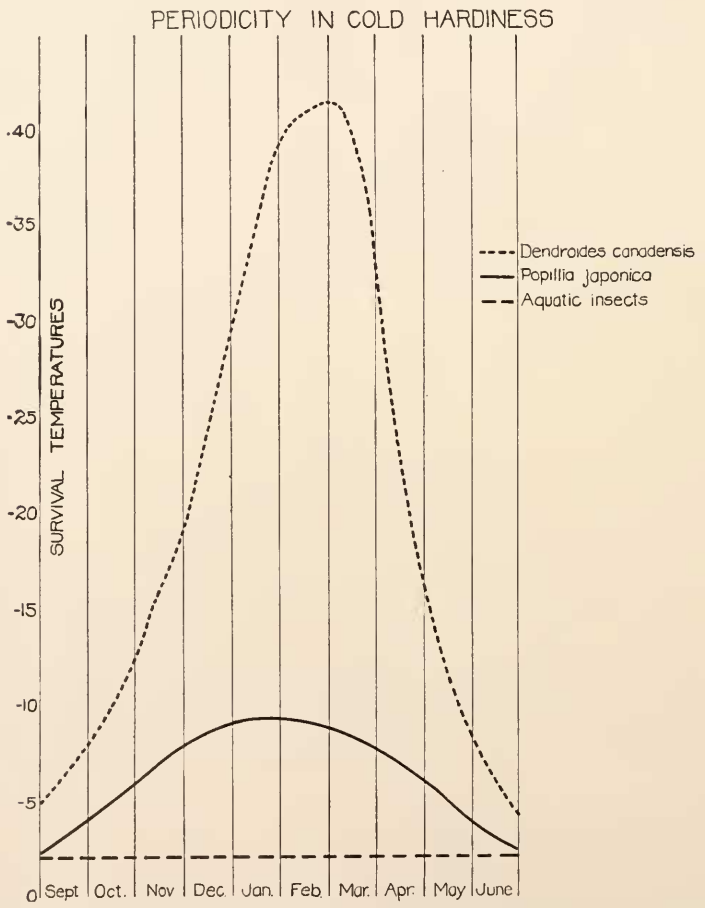

FIG. 5. Periodicity in cold hardiness. *Dendroides canadensis* – – –, *Popillia japonica* ——, aquatic insects — —.

aquatic insects where there is practically none, Payne (1926). This periodicity in cold hardiness is shown in Fig. 5. The relation between undercooling and survival temperatures is shown in Fig. 6. Cold hardiness greater than is usually found in their

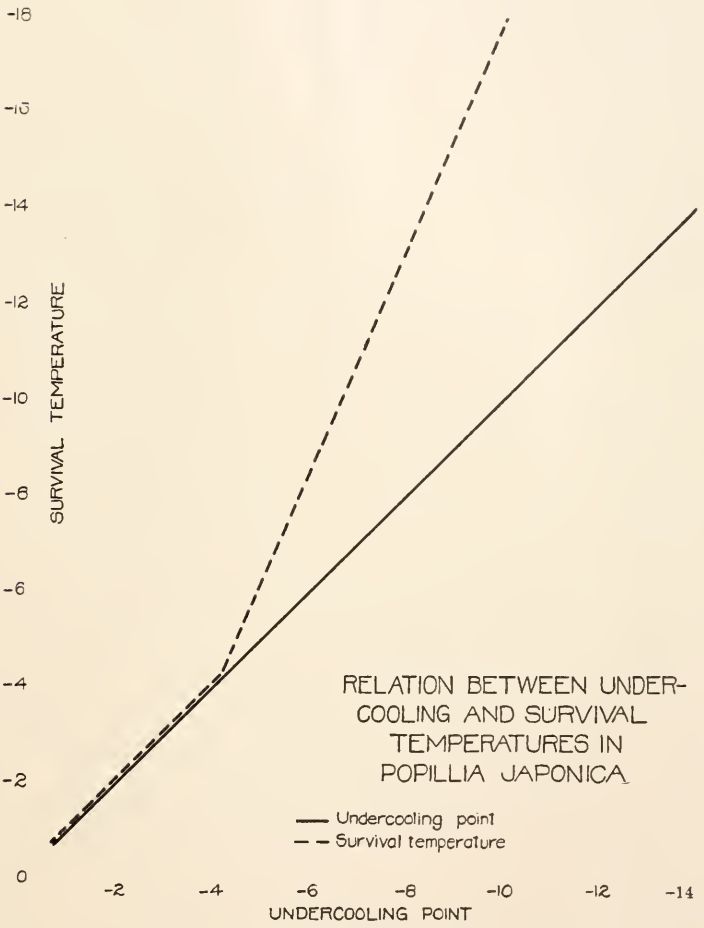

FIG. 6. Relation between undercooling and survival temperatures in *Popillia japonica*. Undercooling point ——; survival temperature — —.

soil habitat can be induced in this insect by dehydration. Conductivity measurements of the blood of dehydrated insects were made. The greatest cold hardiness was found in the dehydrated insects and the least in those infected with wilt disease or

polyhedrälskrankheit. In this disease both the freezing point and the conductivity of the blood approach that of water. In Fig. 3 the conductivities of the Japanese beetle larval blood are plotted against survival temperature. Cold hardiness in this species is more fully measured by conductivity than by either moisture content or undercooling point.

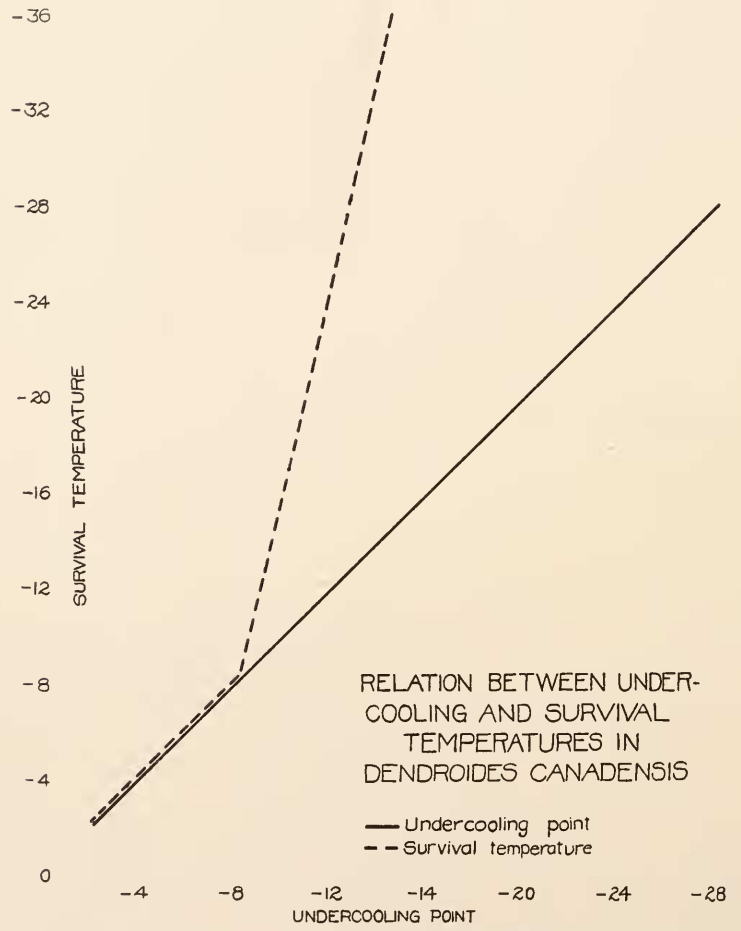

FIG. 7. Relation between undercooling and survival temperatures in *Dendroides canadensis*. Undercooling point ——; survival temperature — —.

Summary.

1. Cold hardiness to the intensity factor of low temperature can be measured by moisture content, undercooling point, and blood conductivity.

2. Up to the time when a given insect can survive freezing, undercooling is a reliable measure of cold hardiness. Beyond the point when an insect can survive freezing, undercooling measures but a part of the total cold resistance of a given insect.

3. Conductivity measurements are found proportional to cold hardiness throughout the whole year. In some insects there is insufficient free body fluid in winter on which to determine blood conductivity.

4. For each species there is a different set of physical constants which measure the cold hardiness of that species.

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COMPENSATORY HYPERTROPHY OF THE TESTES IN BROWN LEGHORNS.[1]

L. V. DOMM AND MARY JUHN.

I. Introduction.

Compensatory hypertrophy of the surviving testis after unilateral castration was observed as early as 1890 by Ribbert (1). Ribbert worked on mammalian material, using young but almost mature rabbits. Ribbert removed the right or the left testis and then compared the surviving gland with controls of the same age after three months; considerable hypertrophy was almost always found. The hypertrophied testis weighed six times as much as one control gland in three cases. There was no difference in the degree of hypertrophy between the right or the left testis. If the operated animal did not increase in weight as much as did the normal controls, then the retained gland also showed a corresponding lack of development.

Lipschütz '22 (2) repeated these experiments of Ribbert, using also rabbit material for his experiments. It appears to follow from Lipschütz's paper that there is a compensatory increase in weight of the surviving testis after unilateral castration when the operation is performed on young rabbits. This increase in size and weight becomes progressively less the longer the operated animals are kept; about one year after the operation there is not much difference in the weight of the surviving testis and in that of one of the control pair of glands. Unilateral castration is not followed by a significant increase in the size of the remaining testis when the gonad is removed in adult rabbits. Lipschütz believes that his results indicate that the testis is incapable of true compensatory hypertrophy; the actual increase in weight observed being due only to a more rapid rate of growth of the isolated gonad.

[1] From the Whitman Laboratory of Experimental Zoölogy of The University of Chicago. The expenses of this investigation were supported in part by the Committee for Research in Problems of Sex of the National Research Council; grant administered by F. R. Lillie.

The two papers cited above report a certain difference in the results obtained. The discrepancy may be due to the time the surviving gonad was retained and also to the age of the animal at the time of operation.

We became interested in the problem of compensatory hypertrophy of the testis as applied to the material used in the laboratory for a variety of experiments, pure bred Brown Leghorn cocks. The experiments were begun early in July 1924 and terminated at the end of April 1925.

We were guided in outlining the course of the experiments by the following points of view: (*a*) the appearance of compensatory hypertrophy as such after the removal of one of the pair of gonads; (*b*) the influence of the age of the birds at the time of the operation on the possible increase in size of the surviving gonad; (*c*) whether the time that the surviving gonad is retained is of effect on the degree of possible hypertrophy taking place. (*d*) Finally we wished to determine if there was a significant difference in the amount of the hypertrophy taking place in the right or the left gonad after unilateral castration.

After the experiments were terminated and the data completed we became acquainted with a paper by Benoît '25 (3). This author carried out a series of unilateral castrations on White Leghorns; the operations were performed on three young birds of 18–20 days of age, on one young bird aged two months and on two birds aged seven months each. Control gonad weights were stated for the groups of different ages. According to the results obtained by Benoît, there is a very real increase in the weight of the surviving gonad when castration is carried out at an early date. In the three cases where unilateral castration was performed in baby chicks the surviving testes were retained for about twelve months. At this date each one of the hypertrophied testes weighed approximately as much as, or slightly more than, both testes of the control. The surviving gonad of the cockerel which was operated upon at two months was retained for seven months, at the end of this time it weighed almost 50 per cent. more than the control pair of testes, but it is important to observe that "controls" of this age vary greatly among themselves. Benoît observed no significant hypertrophy

when unilateral castration was performed on birds aged seven months. The surviving testis was retained about a year. Benoît concludes that there is a hypertrophy of the surviving gonad when the one member of the pair is removed in very young birds; unilateral castration of older birds, after the testes have achieved approximately their normal size is not followed by a compensatory increase in the weight of the surviving gonad.

The results published by Benoît agree on the whole with those obtained in this laboratory, but we differ slightly from him in the observations on older birds as will appear in the discussion.

The phrase "compensatory hypertrophy" defines the conception, viz: that loss results in stimulating the growth of the surviving member to an extent that tends to restore a normal quantitative balance between the total gonad tissue and the bird. It involves the corollary that there is a normal quotient for weight of bird divided by weight of gonad tissue. The present study aims merely at testing this assumption. The difficulties arise from the fact that the assumed normal quotient of weight of bird divided by weight of gonad varies (1) with age very markedly; (2) with the time of year, age being the same; and that (3) no organ of the body probably is so susceptible to general conditions of health as the testis. These difficulties create numerous sources of error for any very exact formulation, so that we felt that it was not desirable in the present status of this subject with reference to our main problems to use a sufficient amount of material and time to reach quantitative results. The present study, although it gives positive results, is therefore merely suggestive.

It is a pleasure to express our thanks to Professor F. R. Lillie for his continued interest in the work and for his helpful suggestions during its course.

II. Data on Unilateral Castration.

All the unilateral castrations were carried out on pure bred Brown Leghorn cockerels that were obtained from one well-known source. The birds were divided into four groups, the first being about one week of age, the second sixteen weeks, the third

twenty-four weeks and the fourth between thirty-two and forty weeks of age at the time of operation.

The operated birds and their controls in each group were hatched at the same time and kept in the laboratory under identical conditions. Comparisons were made only within the groups and in no case between birds of the same age but hatched at different periods.

The cockerels and their controls were weighed at the time of operation and the measurements of the head furnishings and spurs taken. These observations were repeated every eight weeks and a record kept of the condition of the experimental and control birds during the entire experimental period. The testes were removed through an incision between the last two ribs, the gland rapidly weighed and the volume obtained through displacement of normal saline. In the group of baby chicks where the testes were removed at about one week of age, the gonads were not weighed, but the length and width of the gland taken with a pair of fine callipers. The removed testes were fixed in Bouin's fluid at 37° C. and kept in the incubator at that temperature for several hours, varying with the size of the gonad. The usual procedure was followed in washing, etc., and the testes preserved in oil of wintergreen for future histological work.

The greater number of the chicks operated upon at one week of age was lost together with their controls owing to unfavourable weather conditions. The survivors were kept until they were thirty-two weeks of age and then completely caponized or killed.

In the three other groups the right gonad was removed from a certain number of cockerels and the left from a similar number. The surviving right or left testis was then retained for eight, sixteen and twenty-four weeks respectively, at the end of each of these periods one cockerel having a right testis, one cockerel having a left testis and two control birds were completely caponized or killed.

We found in the course of our observations that loss of weight on the part of the cock is reflected in a corresponding diminution of the size of the testes.

The data obtained are outlined in the tables given below. The age of the bird at the time of the operation, the weight of

the bird at the beginning and end of the experimental period is stated. The weights of the removed, hypertrophied and control glands are expressed in grammes and in per cent. of the body weight of the fowl.

In the group A (Table I.), where unilateral castrations were performed on baby chicks aged one week, six operated birds and

Table I.

Results of Unilateral Castration at One Week.

No.	Observations at Time of Operation.			Weighings at 32 Weeks.		
	Chick, Weight.	Removed Testis.		Bird.	Surviving Testis.	
		Length.	Width.		Weight.	Per Cent. Weight.
53.....	45.40	R. 3.5	2.0	1,460.80	L. 19.95	1.35
56.....	54.50	L. 4.1	1.2	1,507.50	R. 7.35	0.48
44.....	67.15	R. 4.5	2.0	1,545.85	L. 13.00	0.83
49.....	66.00	L. 5.0	1.3	992.25	R. 0.85	0.095
46.....	57.95	R. 5.5	1.5	1,275.85	L. 18.70	1.46
50.....	50.90	L. 6.0	1.0	1,048.96	R. 5.10	0.49
62c[1].....	45.00			1,020.60	R. 0.65	0.063
					L. 0.55	0.053
64c.....	46.50			1,602.55	R. 12.80	0.79
					L. 11.25	0.70
65c.....	47.65			1,219.05	R. 6.95	0.57
					L. 7.50	0.61
63c.....	43.00			1,489.15	R. 8.20	0.55
					L. 7.75	0.52
66c.....	44.00			994.25	R. 6.20	0.61
					L. 5.85	0.58

The measurements given for the testes removed at unilateral castration are in mm. All weights are in grammes. Per cent. weight = percentage of weight of testis to total weight of bird.

[1] c = control.

five controls survived for a period of about thirty-one weeks. Unilateral castrations were performed on thirty-five baby chicks and there were a large number of controls; the mortality was due not so much to operative effects but to the very unfavourable weather conditions. Of the six surviving operated birds, three had a left testis while the other three had a right one. Each surviving left gonad is larger than any of the ten control testes; in two of the three cases each surviving left testis is larger than

any of the five control pairs with one slight exception (No.64). These results cannot be due to chance; we are therefore justified in concluding that a surviving left testis, the partner of which is removed at one week after hatching, exhibits a much greater amount of growth than it would have done, approaching in some cases twice the normal growth.

Preliminary histological examinations of sections of hypertrophied testes indicate that all the gonad tissues are equally concerned in this increase in size.

TABLE II.

RESULTS OF UNILATERAL CASTRATION AT 16 WEEKS.

No.	Weighings at Time of Operation.			Later Observations.		
	Bird.	Removed Testis.		Bird.	At 24 Weeks.	
					Surviving Testis.	
		Weight.	Per Cent. Weight.		Weight.	Per Cent. Weight.
819.....	992.25	R. 0.20	0.020	1,687.60	L. 9.60	0.56
820.....	1,077.35	L. 0.35	0.031	1,630.90	R. 6.35	0.38
826c[1]....	1,105.65			1,630.90	R. 8.40	0.50
					L. 9.80	0.60
830c.....	1,162.35			1,602.55	R. 3.00	0.18
					L. 3.10	0.19
					At 32 Weeks.	
815.....	1,048.95	R. 0.36	0.033	1,687.60	L. 30.00	1.81
823.....	907.20	L. 0.17	0.018	1,247.40	R. 15.82	1.26
829c.....	1,460.80			2,071.10	R. 14.11	0.66
					L. 15.78	0.75
831c.....	1,048.95			1,857.70	R. 11.82	0.60
					L. 14.11	0.75
					At 40 Weeks.	
824.....	1,048.95	L. 0.65	0.061	1,574.20	R. 20.18	1.28
825.....	963.90	L. 0.19	0.19	1,517.50	R. 20.25	1.31
833c.....	992.25			1,574.20	R. 11.61	0.73
					L. 14.30	0.90

All weights are in grammes. Per cent. weight = percentage of weight of testis to total weight of bird.

[1] c = control.

The three surviving right testes on the other hand showed no such increase in size, and in fact did not differ significantly from a single control testis.

The second group of young cockerels was castrated at sixteen weeks and the results are tabulated in Table II. The surviving testes were retained eight, sixteen and twenty-four weeks respectively. It was originally planned to observe the degree of compensatory hypertrophy to forty-eight weeks at which time the birds have been fully mature for about sixteen weeks. However a number of birds died owing to one cause and another so that the last data were obtained on cocks aged forty weeks and only on two right testes.

There is no observable compensatory hypertrophy either of the right or the left testis when the glands were retained only for eight weeks after the operation. There was an increase in size during this period but this was identical with the control glands. After a period of sixteen weeks, however, both the left and the right surviving testes show a considerable degree of compensatory hypertrophy, the left testis being heavier than both testes together of each of the two control pairs; the right testis on the other hand, while it exhibited a high percentage weight, was only slightly heavier absolutely than a single control testis. Three birds were available for observation after twenty-four weeks, two of them having a right testis each while the third served as control. Each of the two right testes weighed about 75 per cent. as much as the control pair, the degree of compensatory hypertrophy on a percentage basis being similar to the amount observed after sixteen weeks.

Table III. gives the data for the next group. The cockerels in this group were unilaterally castrated at twenty-four weeks and then observed to forty-eight weeks at intervals of eight, sixteen and twenty-four weeks, respectively. After eight weeks the surviving left testis showed a certain degree of hypertrophy, weighing much more than one of the control pair of gonads and only slightly less than the other. The right testis had not increased at all as compared with the normal. The same is true after sixteen weeks; the left surviving gland is larger than any one testis of the control pairs but not as heavy as one of the

Table III.

Results of Unilateral Castration at 24 Weeks.

No.	Weighings at Time of Operation.			Later Observations.		
	Bird.	Removed Testis.		Bird.	At 32 Weeks.	
					Surviving Testis.	
		Weight.	Per Cent. Weight.		Weight.	Per Cent. Weight.
810	1,574.20	R. 0.45	0.027	1,801.00	L. 10.90	0.60
801	1,332.45	L. 0.20	0.016	1,715.95	R. 0.65	0.041
901c[1]				1,574.20	R. 1.50	0.095
					L. 1.31	0.093
902c				2,241.20	R. 6.65	0.29
					L. 8.95	0.39
					At 40 Weeks.	
813	1,020.60	R. 0.25	0.024	2,127.80	L. 17.05	0.80
804	1,545.85	L. 5.0	0.32	2,241.20	R. 8.60	0.37
809c				2,212.85	R. 12.70	0.57
					L. 14.60	0.65
907c				2,099.45	R. 7.70	0.36
					L. 8.0	0.38
					At 48 Weeks.	
808[2]	963.90	R. 0.20	0.020	1,162.35	L. 3.18	0.27
812	1,573.20	L. 2.70	0.17	1,212.85	R. 10.83	0.83
904c				2,127.80	R. 13.23	0.62
					L. 11.81	0.55
905c				2,042.75	R. 4.15	0.20
					L. 3.45	0.11

All weights are in grammes. Per cent. weight = percentage of weight of testis to total weight of bird.

[1] c = control.

[2] No. 808 was ill during the first half of the experimental period and lost considerable weight which it had not regained at the time the bird was killed and the surviving testis removed.

pairs, while the right surviving testis is not as heavy as control single testes. After twenty-four weeks there was no hypertrophy at all to be observed in the left surviving testis; as this bird had been in poor condition and lost considerable weight during the experimental period, we do not attach much significance to

this case. The right surviving testis is probably to be regarded as hypertrophied, being heavier than one of the control pairs and its percentage weight greater than any one testis of the other control pair.

In the group where the cocks were mature (Table IV.), there was not so much difference to be observed in the hypertrophy of the surviving testes. The left testis had increased relatively more in size compared with the gland removed at the operation than the right testis. The hypertrophying testes were retained

Table IV.

Results of Unilateral Castration at 32–40 Weeks.

No.	Weighings at Time of Operation.			Weighings at 40–48 Weeks.		
	Bird.	Removed Testis.		Bird.	Surviving Testis.	
		Weight.	Per Cent. Weight.		Weight.	Per Cent. Weight.
121.....	1,574.20	R. 3.95	0.25	2,042.75	L. 13.87	0.67
114.....	1,517.50	L. 6.55	0.43	1,659.25	R. 14.27	0.86
123c[1]....	1,517.50			1,517.50	R. 7.36	0.48
					L. 7.52	0.49
128c.....	1,829.35			1,687.60	R. 5.0	0.39
					L. 4.93	0.39

All weights are in grammes. Per cent. weight = percentage of weight of testis to total weight of bird.

[1] c = control.

for only eight weeks, the observation period being planned to extend only to forty-eight weeks of age. The left surviving testis was larger than any single testis of the two control pairs, but not as heavy as either pair together. The right surviving testis was heavier than one control pair and almost equal to the other pair, being 0.86 per cent. of the body weight while the two control testes pairs weighed 0.78 per cent. and 0.97 per cent. respectively. While the cases are few, the evidence seems to indicate compensatory hypertrophy in this group also after a very short period.

IIa. Summary of Results of Unilateral Castration at Various Ages.

The preceding tables and descriptions demonstrate that removal of one of the testes pair induces an increase in size of the retained partner when the retention period is of sufficient length, with the exception of the right isolated testes of young chicks (Table I.).

When unilateral castration is performed on young cockerels the left surviving gonad hypertrophies to a greater degree than does the right one, the one differing result obtained in No. 808 being due to the bird's general condition.

The increase in weight of the isolated testis becomes manifest only after a certain interval following castration, the interval being apparently somewhat longer for the right hypertrophying testis than for the left one when younger birds are operated upon.

Table V.

Data Compiled from Tables I.–IV. Control Testes Weights Stated as Averages.

Age of Bird.		Weight of Isolated Testis.	Average Weight of Controls.	
At Operation.	At Removal.		Testis Pair.	Single Gland.
One week.....	32 weeks	L. 13.00 L. 18.70 L. 19.95 R. 0.85 R. 5.10 R. 7.35	13.54 (5p.)	6.77
16 weeks.....	24 weeks	L. 9.60 R. 6.35	12.15 (2p.)	6.07
	32 "	L. 30.00 R. 15.82	27.91 (2p.)	13.98
	40 "	R. 20.18 R. 20.25	25.91 (1p.)	12.85
24 weeks.....	32 weeks	L. 10.90 R. 0.65	9.10 (2p.)	4.80
	40 "	L. 17.05 R. 8.60	21.50 (2p.)	10.75
	48 "	L. 3.18 R. 10.83	16.32 (2p.)	8.16
32–40 weeks.....	40–48 weeks	L. 13.87 R. 14.27	12.40 (2p.)	6.20

All weights are in grammes.

The latent period is much shorter when adult birds are unilaterally castrated and here the right isolated testis hypertrophies at the same rate and in the single case available even to a slightly greater degree than the left (Table IV.).

Comparison with the averages of the controls rather than with single controls as set forth in the tables increases the probability of these conclusions as examination of Table V. shows. Individual cases emphasize these general conclusions (Table I., Nos. 53, 44, 46. Table II., Nos. 815, 824, 845. Table IV., Nos. 114, 121).

Lipschütz's suggestion for mammals that unilateral castration produces only a more rapid rate of growth rather than a definitive compensatory enlargement of the surviving gonad does not seem a probable interpretation of our results. It would mean that there would be no actual plus in weight of the isolated gonad over one of the control testes pair at the end of the developmental period of the glands. Such a statement requires a definition of the endpoint of growth of the testes, and in view of the normal variation in the weight of the testes as well as the seasonal variation the feasibility of such an absolute determination appears questionable.

The cocks are mature at thirty-two weeks and compensatory hypertrophy as defined is demonstrated not only at this time but as late as forty-eight weeks.

III. Normal Size Relations of Right and Left Testes.

During the course of the experiments we accumulated some data on the size of the right and the left testes of normal cocks. The majority of the observations were made on the gonads of different birds but the records of the control birds in the preceding tables are for pairs. Tables VI. and VII. give the measurements obtained in one week old chicks. The length and width of the right and left testes are given as it was impracticable to secure accurate weights. The weights of the chicks are also stated for comparison. There appears to be a very slight advantage in size on the part of the left testes at this age. In older birds we find such an individual variation occurring in birds of the same age and even of approximately

TABLE VI.

MEASUREMENTS OF LEFT TESTES REMOVED FROM CHICKS AGED ONE WEEK.

No.	Testes.		Weight of Chick.
	Length.	Width.	
51	4.4 mm.	1.5 mm.	45.15 gs.
61	5.1 "	2.0 "	55.30 "
62	4.0 "	1.8 "	45.0 "
0	3.5 "	1.1 "	39.30 "
63	5.0 "	1.2 "	43.0 "
64	4.5 "	2.0 "	46.50 "
65	3.0 "	2.0 "	47.65 "
56	4.1 "	1.2 "	54.50 "
64A	4.0 "	1.1 "	40.40 "
66	4.0 "	1.5 "	44.0 "
67	5.0 "	1.5 "	49.50 "
68	5.0 "	1.9 "	44.75 "
69	4.8 "	1.9 "	49.90 "
50	6.0 "	1.0 "	50.90 "
49	5.0 "	1.3 "	66.0 "
48	5.3 "	1.3 "	57.85 "
40	4.5 "	1.5 "	52.80 "
47	5.2 "	1.7 "	57.85 "
39	5.0 "	1.3 "	63.80 "

No. of Cases.	Average of Measurements of Testes. Length.	Width.
19	4.6 mm.	1.5 mm.

TABLE VII.

MEASUREMENTS OF RIGHT TESTES REMOVED FROM CHICKS AGED ONE WEEK.

No.	Testes.		Weight of Chick.
	Length.	Width.	
52	3.5 mm.	1.0 mm.	43.75 gs.
54	4.3 "	1.1 "	43.87 "
53	3.5 "	2.0 "	45.40 "
55	3.0 "	1.5 "	42.60 "
57	5.0 "	1.5 "	49.0 "
58	4.0 "	1.0 "	43.30 "
59	4.0 "	1.3 "	45.60 "
61	5.1 "	2.0 "	55.30 "
70	4.0 "	1.5 "	39.90 "
46	5.5 "	1.5 "	57.95 "
44	4.5 "	2.0 "	67.15 "
43	6.7 "	1.5 "	56.30 "
42	6.0 "	1.5 "	51.10 "
41	5.0 "	1.5 "	59.0 "

No. of Cases.	Average of Measurements of Testes. Length.	Width.
15	4.5 mm.	1.2 mm.

identical weight, that valid conclusions cannot be drawn from the data obtained in different cocks. The tables compiled are omitted for this reason.

Where testes of one pair were observed as was done for the control cockerels (Tables II.–IV.) the left testes were larger than the right in one case out of two at twenty-four weeks. At thirty-two weeks, the left testes were larger in two out of three pairs, while at forty-eight weeks, the left testis was very slightly heavier (0.01 per cent.) than the right one in one pair; it was smaller than the right testis in two pairs and finally there was one pair in which the gonad weighed exactly the same amount on the left and on the right side.

The tendency of the left testis to be rather larger than the right one in embryonic chicks has been observed by a number of authors. Firket, '14 (4), states that the right testis is noticeably smaller than the left one in the chick at the seventh day of incubation and quotes Semon, '87 (5), as saying that the left testis is much larger at the beginning of its development.

According to Swift, '16 (6), the left embryonic testis is noticeably larger than the right one in the five day chick and the germinal epithelium of the left gonad is also thicker and more extensive. This difference in favor of the left testis is also visible in the six and nine day chick. Riddle, '16 (7), finds no difference between the right and the left testes in common fowl, the age of the birds is not stated.

The greater tendency towards hypertrophy of the left testis discussed under II.*a*, is presumably associated with this embryonic condition, and is of interest in comparison with the very pronounced asymmetry of the female.

IV. Discussion.

From the results described in the preceding pages as well as from the experimental data published by Benoît, the occurrence of compensatory hypertrophy following unilateral castration in young male fowls seems to be well established. We found compensatory hypertrophy of the retained gonad also in adult cocks, differing in this point from Benoît's observations. The period during which the surviving testes were permitted to

hypertrophy was shorter in our cases than in those reported by Benoît and it is possible that the explanation for the different results obtained may be found in this fact.

The problem of compensatory hypertrophy of the gonad presents its teleological as well as its physiological aspects. The term itself has teleological implications; from this point of view the "purpose" might be either to provide increased reproductive capacity, which appears unnecessary, or to establish a balance of hormones. There again the solution is unsatisfactory for less than one testis is sufficient for maintenance of sex characters as shown by Pézard, '21 (8); '25 (9); Champy, '25 (10).

Physiologically considered it would appear to be obvious that the growth of testis tissue is balanced against something else in the organism.

The general bodily metabolism favors the growth of a definite amount of gonad tissue and no more. The removal of one testis of a pair leaves a balance of conditions favorable to the continued growth beyond its normal size of the surviving member which thereupon responds in proportion to its growth capacity up to the limits of the favorable metabolism. When unilateral castration is performed very early this may result in a single testis greater in weight than a normal pair (Table I., case 16, Table II., case 815), whether there may be a progressive limitation of capacity for compensatory growth with increasing age as maintained by Benoît is still an open question as far as our own results are concerned.

No theory is put forth in explanation of the change in the reaction between gonad and organism which follows removal of one of the gonad pair; it is shown by the facts. But the importance of the principle appears again in the transformations of the female following ovariotomy. The right rudimentary gonad responds with a proliferation of the kind of tissue of which it is composed at the time the demand on it is created, thus producing the various types of right compensatory growth described in completely and incompletely castrated hens by Domm, '24 (11); '27 (12). The principle of compensatory hypertrophy is also illustrated in the growth of grafts.

V. Summary.

1. Unilateral castration in Brown Leghorn cockerels is followed by compensatory hypertrophy of either the right or the left retained testis when the operation is performed on birds aged 16, 24 and 32–40 weeks.

2. The removal of the right or the left testis in chicks aged one week caused a compensatory hypertrophy of the left retained gonads only, after a period of thirty-one weeks, in our experiments (Table I.).

3. There may be a certain period before increase in weight of the retained gonad over the controls becomes manifest; the length of this period is variable.

4. The left retained testis shows a greater tendency towards hypertrophy than does the right.

5. There seems to be a difference in the weight of the left and the right normal testes; this is in favor of the left gonad in very young birds and then gradually seems to become shifted to the right testis as the bird becomes older and reaches maturity.

6. A tentative suggestion is made, that there may be some relation between the greater amount of germinal epithelium in embryonic left testes and the greater tendency towards hypertrophy of the left surviving gonad which is particularly manifest when unilateral castration is performed on young birds.

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NOTE ON THE HEMOLYTIC ACTION OF SEBRIGHT SERUM ON LEGHORN CORPUSCLES.[1]

MARY JUHN.

In a series of experiments on grafts of Leghorn testes into Sebright capons and Sebright testes into Leghorn capons carried out by Mr. Roxas in this laboratory (1), the observations were made that the Leghorn testis takes readily in the Sebright but that the inverse is not the case. A much greater mortality was also observed in the Leghorn hosts having Sebright grafts than in the Sebrights having Leghorn grafts. In actual figures:

	Leghorn Testis into Sebrights.	Sebright Testis into Leghorns.
No. of birds	38	50
Died from known causes	12, 31%	8, 6%
Died, cause unknown	8, 21%	24, 48%
Survived to end of exp.	18, 47%	18, 36%
Birds with succ. grafts (% of surviving birds)	11, 60%	3, 8%

According to a verbal communication by Mr. Roxas, the Leghorn capons with Sebright grafts that succumbed, showed evidences of pronounced anæmia, this being evident in the lack of color of the headfurnishings which became progressively almost a dead white.

In attempting to determine the causes for the greater mortality among the Leghorn hosts as well as the reason for the much smaller per cent. of successful grafts of Sebright testes into the Leghorns, the following suggestions present themselves. Considering first the conditions in the Sebrights having Leghorn testes grafts, the per cent. of takes is high; the mortality which may be attributed to the graft (cause of death unknown, 21 per cent.) is relatively not so pronounced. We may believe then that the Leghorn tissues are readily incorporated in the

[1] From the Whitman Laboratory of Experimental Zoölogy of the University of Chicago. The expenses of these investigations were supported in part by the Committee for Research in Problems of Sex of the National Research Council; grant administered by F. R. Lillie.

Sebright organism, they soon become vascularized, and being supplied with nutritive substances, persist and even show active growth. There is no extended necrosis in the Leghorn grafts beyond some presumably occurring when it is first implanted and previous to vascularization.

When Sebright testes are implanted into Leghorn tissues however, there is a relatively high percentage of mortality attributable to the graft (cause of death unknown, 48 per cent.) and a very low percentage of takes, 8 per cent. of the surviving birds and only 6 per cent. of all the birds operated upon. There is no evidence of vascularization of the Sebright testis grafts, on the contrary the appearance of the Leghorn capons after Sebright implantations have been made lead one to believe in a greater activity of the lymphocytes and a subsequent elimination of the destroyed graft tissues into the blood stream of the host. Blood counts before and after grafting as well as control of the body temperature would presumably serve to determine the accuracy of these assumptions. The resorption of the graft in a large percentage of cases with subsequent mortality of the host from one cause or another is however apparent.

There is an extensive literature on the subject of the toxicity of organ extracts when injected intraperitoneally, subcutaneously or intravenously. The intravenous injections produce the most rapid lethal effects, but subcutaneous injections of organ paste into guinea pigs, were reported by Brieger and Ulenhuth (2) to kill the animals within 24 hours after the injection. This effect was correlated by Dold and Kodama (3) with the toxic action of tissues in a state of destruction and with the causes of death after burns. Pfeiffer (4) states that in acute cases of death from burns, the cause of the mortality may be traced to toxic poisoning induced by protein fission products. These products appear in excessive quantities owing to the resorptive destruction of the proteins which have been changed and killed through heat.

It appears reasonable in view of the findings in comparable fields reported above, to correlate the percentage of deaths in the Leghorns having Sebright grafts with the non-success of these grafts. The continuous resorption of the graft tissues, may, and probably does, set free into the blood stream of the

host toxic substances resulting from the destruction of the implant, and these toxic substances finally prove fatal to the carrier of the graft.

The point of interest is the reason for the different results obtained in these cross-transplantations. The possibility of some specific differences in the blood of the two breeds of fowl was suggested by Prof. F. R. Lillie; the tests made to establish the presence of such differences are described in the latter part of the paper.

My thanks are due to Professor Lillie both for suggesting the problem reported here and for his continued helpful interest in the work.

Some experiments carried out by Sokoloff (5) serve to illustrate that such conditions can be found in other vertebrates. Sokoloff working on homotransplantations in rabbits found the presence of three types of blood and states that grafts only take when made into animals of identical blood constitution with the donor. When grafts are made into animals where the serum of the host agglutinates the corpuscles of the donor, the graft shows complete necrosis. This destruction of the graft cells leads to the production of specific antibodies in the host which have an unfavorable action on the graft. Any graft will cause the formation of some percentage of antibodies but these are counteracted by the graft as soon as vascularization is established and they then disappear from the blood stream.

Furthermore according to Sokoloff, immunization by intraperitoneal injections of an emulsion of the organ to be grafted causes the appearance of antibodies as well, and the presence of these antibodies inhibits take and growth of the graft.

In studying the conditions in the Leghorns and Sebrights, no tests were made for the determination of circulating antibodies. The sera of Leghorn and Sebright cocks and capons were tested for their agglutinating and hemolytic action on the corpuscles of all four kinds of birds.

The experiments were repeated four times, the preparation of the serum and the corpuscle suspension being identical in every case. For the corpuscles blood was drawn from the ventricle into a syringe moistened with a 1.5 per cent. sodium citrate

solution, 1 cc. of blood was injected into 19 cc. of 1.5 per cent. sodium citrate, the corpuscles then washed four times, centrifuging at low speed to just sediment the corpuscles and the final suspension being brought to 5 per cent. in normal saline. For the serum, blood was run into small test tubes and kept at room temperature over night.

The serum was diluted for all the experiments 1 : 4 with normal saline; the corpuscle suspension being 5 per cent. as stated above. Agglutination tests were made in the hanging drop according to the method outlined by Ascoli (6). No agglutination was observed in any of the serum-corpuscle combinations.

In the tests for the possible hemolytic action of the sera of any one of the four birds, sixteen hemolysis tubes were used in each experiment. Four of the tubes were controls, having the own corpuscles added to the serum, the other twelve were all the possible combinations. One cc. of the 5 per cent. corpuscle suspension was rapidly run into 1.5 cc. of the serum dilution. The tubes were then shaken, placed in the incubator at 38.5° C. for two hours and shaken again every quarter of an hour during this interval. The tubes were then placed in the ice-box at + 9° C. over night and observed the following morning. Furthermore a 5 per cent. corpuscle suspension in saline was always preserved to the end of the period of observation.

The table given demonstrates the results obtained more clearly than any written description. The greater tendency of the Sebright cock and capon serum to hemolyze the Leghorn corpuscles may be noted, but attention must be drawn to the fact, that this is not a constant phenomenon and that in the case of the Sebright capon serum the own corpuscles are hemolyzed to approximately the same degree.

In a single experiment not recorded in the table, the sera of all the birds was diluted 1 : 40 with normal saline. At this degree of dilution distinct hemolysis was observed in the tube having Sebright capon serum and Leghorn capon corpuscles. There was not even a trace of hemolytic action to be noted for any of the other serum-corpuscle combinations.

The different action of the sera tested does not prove an actual difference in the tissues but it is of interest to note the

SERUM.

	Sebright ♂.				Sebright Capon.				Leghorn ♂.				Leghorn Capon.			
Corpusc.	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
Sebr. ♂	−	+?	−	−	++	o	++	++	+?	+?	−	+	+?	+?	−	−
Sebr. c	o	o	−	−	+++	o	++	++	+	+?	−	++	+	++	−	−
Legh. ♂	+++	+++	−	−	++	o	+	++	+?	−	−	−	+?	+?	−	−
Legh. c	+?	+?	−	−	+?	o	++	+++	+	+?	−	+	−	+?	−	−

1, 2, 3, 4 are the four separate experiments referred to in the text.
o = no tube. − = no hemolysis. +? = very faint traces of hemolysis. + = hemolysis distinct. ++ = hemolysis definite. +++ = entire tube hemolyzed.

parallel between the hemolytic action of some of the Sebright sera on Leghorn corpuscles and the non-success of Sebright grafts in Leghorns except in a very small percentage of cases.

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CONFIGURATIONS OF BIVALENTS OF HYACINTHUS WITH REGARD TO SEGMENTAL INTERCHANGE.

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INTRODUCTION.

In the majority of the flowering plants examined by the writer, and apparently also in most of those investigated in this respect by others, the homologous chromosomes, which form bivalents at the reduction metaphase, are joined only at the extreme ends. As examples, Canna and Datura may serve, in which this rule holds in the triploids as well as in the diploids. In the largest bivalent of *Uvularia*, however (Belling, 1926), there are additional points of junction (nodes) not at the ends. The short and medium chromosomes of *Uvularia* seem usually to be connected at or near the constriction, and the same is the case with the short and medium chromosomes of *Hyacinthus* (Belling, 1925). These will not be further considered here.

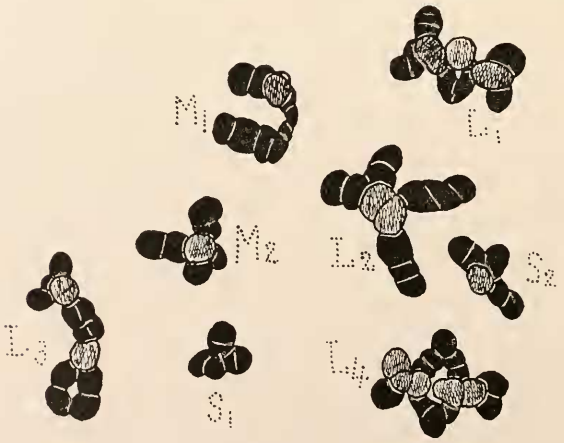

FIG. 1. Camera drawing of the eight bivalents of the diploid hyacinth, squeezed from a pollen-mother-cell. The four large bivalents are alone considered here. They are described in the text.

But the four large bivalents of *Hyacinthus* show, like the large bivalent of *Uvularia*, many connections not at the ends (Fig. 1). It has been pointed out in regard to *Uvularia* (Belling, 1926) that the simplest hypothesis is that these connections (nodes) not at the ends represent places where two of the four chromatids have undergone segmental interchange by fracture and recombination. In *Hyacinthus* it can apparently sometimes be seen with the microscope that two of the four chromatids are bent back at a node, so as to continue along the same sides of the bivalent (Fig. 3). It has also been shown that the homologous chromosomes of the rings and V's formed by the large bivalent of *Uvularia*, acted when separating as if they were not merely twisted across one another, but had undergone a process which produced some interlacing of chromatids at the nodes. This would prevent the simple untwisting of the homologues at the anaphase, and such untwisting has been shown not to occur in *Uvularia* (Belling, 1926). This would also lead (as has been abundantly shown by Janssens, 1924, and others, in animals) to the separation of whole upper and lower halves of vertical rings and V's, and to one chromatid passing up and one down from both sides of horizontal rings or V's, which may get smaller as the process advances, without opening up. This is what takes place in *Uvularia*, and apparently also in *Hyacinthus*.

In *Hyacinthus*, as already stated, the homologues are not always connected at one or both of the extreme ends, but are connected at other places (nodes). This is especially the case with the four long chromosomes. Hence a study of these may show, by the nature of their configurations and their mode of separation at the reduction metaphase, whether the nodes correspond to what would be expected if they were due to segmental interchange between chromosomes (crossing-over of genes).

If the nodes in the long bivalents of the hyacinth are due to segmental interchange, the following phenomena should be observable: (1) the nodes should occur at *different points* in the bivalents in different cases; (2) these nodes should be at equal distances from the ends of both homologues; (3) the nodes should be visible at the late prophase (diakinesis stage or earlier) as well as at the metaphase; (4) the horizontal rings or V's

should split into separate chromatids, while the vertical rings or V's should divide into upper and lower halves, without separating into chromatids; (5) the numbers of bivalents with one or two nodes should permit of a calculation of the numbers of chromatids with segmental interchange at no, one, or two points, which should possibly more or less resemble the occurrence of no, single, or double crossing-over in *Drosophila*. In such a calculation, the numbers of chromatids with no segmental interchange would be equal to twice the cases of single nodes plus the number of cases of double nodes. The total number of chromatids with one point of interchange would be got by adding twice the number of bivalents with single nodes to twice the number with two nodes. While the chromatids with two points of interchange are equal to the number of bivalents with two nodes. It should be possible to test these five points.

(It seems obvious that a junction of homologous chromosomes at the ends has no relation with segmental interchange. It is probably otherwise with junctions at the point of constriction of the chromosome, where segmental interchange may well take place.)

It was for the purpose of testing this hypothesis that the present study was made.

Large Bivalents of *Hyacinthus*.

The variety of *Hyacinthus orientalis* investigated was one of those formerly studied (Belling, 1925), and was chosen because it could be readily identified by the flowers, and had marked characters even in the bulbs. This was the diploid clone called "Yellow Hammer." The bulbs were obtained in October, and put into water during that month and the next. Division of the pollen-mother-cells usually accompanied the development of the first roots. The pollen-mother-cells were instantaneously fixed by being squeezed out from the anthers into iron-acetocarmine. The chromosomes were observed with Zeiss' water-immersion objective 70, yellow-green light, and a water-immersion condenser.

At the first metaphase in the pollen-mother-cells (Fig. 1) the four long chromosome pairs commonly assume one of six different

configurations (Diagram I.). Three of these are shown in Fig. 1, where the cross (L_2) is near the center, two single rings with

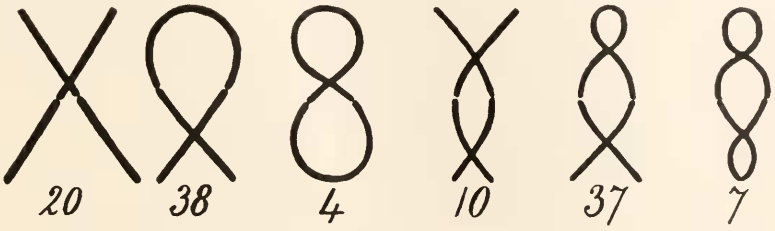

DIAGRAM 1. Numbers of different configurations of large bivalents. The cross, single ring and V, and figure of 8, have one node; while the ring and two V's, the double ring and V, and the triple ring have 2 nodes.

double V's are on the right (L_1 and L_4), and a double ring and V on the left (L_3). Four forms in the late prophase are shown in Fig. 2. They are: (*a*) the ring and V; (*b*) the double ring

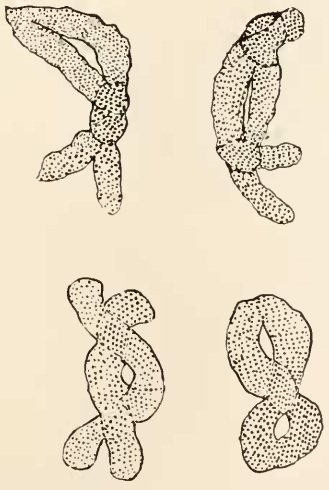

FIG. 2. Four camera drawings of large bivalents at the late prophase. (This stage is difficult to obtain.)

and V; (*c*) the ring and two V's; and (*d*) the figure of 8. The cross and the triple ring were not found free, the bivalents being usually clustered at this stage. Several configurations are drawn in Fig. 3. It may be specially noticed that the four examples of the cross shown in Fig. 3 have the junction in different positions with regard to the chromosome ends. The cross in the middle

has arms which taper to the center, the two homologues having apparently joined at the constrictions. The last two drawings in the lowest line of Fig. 3 are the same bivalent at two different focusses, apparently showing one transverse and one reflexed

FIG. 3. Camera drawings of eight bivalents. The four crosses have the nodes at different points, but always at equal distances from the ends of the homologues. The lowest arm of the second cross in the second line is much foreshortened. The last two figures show the effects of change of focus on the node.

chromatid of one homologue, the same thing being often observable at a different focus in the other homologue also. These are pulled out by the spindle fibers which are attached at the apex of the bend at the median constriction.

One hundred and sixteen of the long bivalents were classified in six groups (Diagram 1), mostly after squeezing chromosomes and cytoplasm from the cell. The results were (Diagram 1): 38 cases of the ring and V; 20 X's; and 4 figures of 8 (totalling 62 with one node); 37 cases of the double ring and V; 10 cases of the ring and two V's; and 7 cases of a triple ring; totalling 54 cases with two nodes. No bivalents with triple nodes were certainly demonstrated. If only two chromatids underwent segmental interchange at any one node, as seems to be indicated

by the microscopical phenomena, we have for the resulting pollen grains and any particular long chromosome, on the hypothesis of segmental interchange: no segmental interchange (124 + 54), 178; single point of interchange (124 + 108), 232; and double interchange, 54; out of a total of 464 chromosomes or pollen grains.

This gives in percentages: chromosomes with no interchange 38 per cent.; chromosomes with single interchange, 50 per cent.; and chromosomes with double interchange, 12 per cent. The ratio of single to double interchange on the hypothesis is thus 4.3 to 1.

This is not far from the numbers of no, single and double points of crossing-over given for the first chromosome of *Drosophila melanogaster* by Morgan (1925). The second and third chromosomes of *Drosophila*, however, seem to differ in this respect.

In Figs. 4 and 5, there are examples of the separation of chromatids and homologues which tend somewhat to prove the hypothesis of segmental interchange. In Fig. 4, L_1 is especially instructive. Here on one side of the constriction (and spindle fiber attachment) there was apparently a horizontal ring, and

FIG. 4. Camera drawing of the bivalents in a cell in which the four large ones had not completely separated, the drawing paper being shifted after each was drawn. Some are foreshortened, especially L_2 and L_4.

on the other side perhaps a vertical ring and a small V; or only a large vertical V. The horizontal ring evidently split into two ring chromatids, while the vertical ring remains. In L_3 the constriction is in the vertical ring. (L_2 and L_4 show nothing more.) In Fig. 5, L_1 shows apparently the separation of the

FIG. 5. Slightly more advanced stage than Fig. 4.

parts of a cross, the two horizontal members of which can be seen splitting into chromatids, one passing to each pole.

DISCUSSION.

The following points seem most worthy of notice.

(1) At some nodes it appears as if both chromatids of each homologue could be seen, one obliquely transverse, and the other reflexed. The spindle fiber is often attached (at or) near the node also at the median constriction.

(2) Some rather scanty data seem to show in *Hyacinthus*, what is more abundantly demonstrated in *Uvularia*, namely, that single chromatids of each homologue pass to the poles from both sides of horizontal rings and V's, and that the two chromatids of each homologue remain connected in the separate halves of vertical rings and V's. So the horizontal rings and

V's diminish in size as their chromatids are pulled out by the spindle fibers towards the poles.

(3) The numbers of cases of possible segmental interchange in the chromatids of the large bivalents, calculated from the numbers of nodes, agrees roughly with the numbers of cases of crossing-over found in the first chromosome of *Drosophila*, which is especially favorable for this study.

Some process of segmental interchange seems demanded by the genetic evidence in *Drosophila*, *Zea*, *Lathyrus*, and the other plants and animals which have shown cases of crossing-over. Hence it is apparently the natural scientific procedure to accept segmental interchange as a working hypothesis to account for the nodes and internodes of the chromosome pairs in the *Orthoptera* and other animals, and also in *Uvularia* and *Hyacinthus*. This is the more imperative in that there seems no other available working hypothesis.

Summary.

(1) The four large bivalents of *Hyacinthus* show in 62 cases one node, and in 54 cases two nodes where the homologues cross.

(2) At these nodes it can apparently be seen with the microscope that one chromatid of each homologue passes obliquely across, while the other seems bent back along the other homologue.

(3) The hypothesis of previous segmental interchange at such a point is assumed until a better hypothesis is found.

(4) The numbers of chromatids showing such points of segmental interchange, according to the hypothesis, calculated from the 116 bivalents examined, were 38 per cent. with no interchange, 50 per cent. with one point of interchange, and 12 per cent. with two points of interchange.

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